

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/19, C07K 14/52, 16/24, C12N 1/21, 5/10, 1/19, A01K 67/027, A61K 38/19, C12Q 1/68, C12N 15/11</b>	<b>A1</b>	(11) International Publication Number: <b>WO 95/21920</b> (43) International Publication Date: 17 August 1995 (17.08.95)
--	-----------	---

(21) International Application Number: **PCT/US94/08806**(22) International Filing Date: **5 August 1994 (05.08.94)**

## (30) Priority Data:

08/196,025	14 February 1994 (14.02.94)	US
08/203,197	25 February 1994 (25.02.94)	US
08/215,203	21 March 1994 (21.03.94)	US
08/252,491	1 June 1994 (01.06.94)	US

(71) Applicants: **ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). UNIVERSITY OF WASHINGTON [US/US]; Seattle, WA 98195 (US).**(72) Inventors: **HOLLY, Richard, D.; 12539 10th Avenue NW, Seattle, WA 98177 (US). LOK, Si; 806 NW 52nd Street, Seattle, WA 98107 (US). FOSTER, Donald, C.; 3002 NE 181st Street, Seattle, WA 98155 (US). HAGEN, Frederick, S.; Christoffers Alle 139, DK-2800 Lyngby (DK). KAUSHANSKY, Kenneth; 19745 NE 127th Place, Woodinville, WA 98072 (US). KUIJPER, Joseph, L.; 6640 NE 154th Street, Bothell, WA 98011 (US). LOFTON-DAY, Catherine; 23908 35th Avenue West, Brier, WA 98036 (US). OORT, Pieter, J.; 915 N. 198th, Seattle, WA 98133 (US). BURKHEAD, Steven, K.; 800 NE 66th Street, Seattle, WA 98115 (US).**(74) Agent: **PARKER, Gary, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).**(81) Designated States: **AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).**

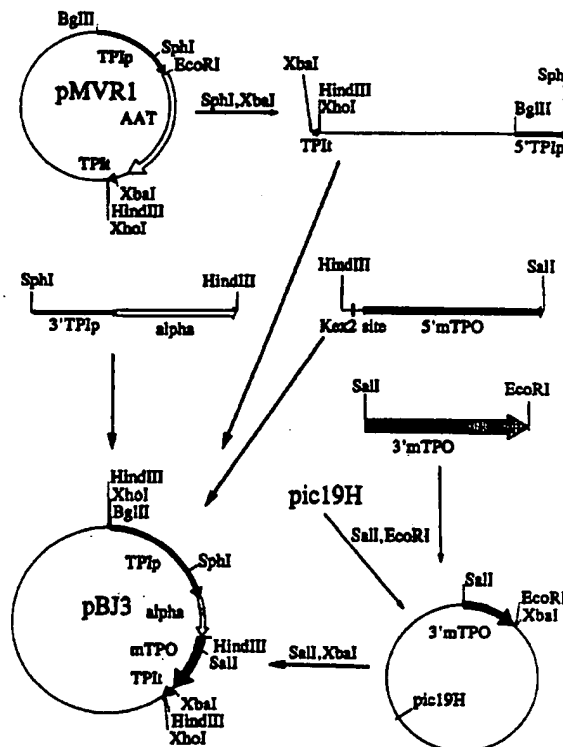
Published

With international search report.

(54) Title: **HEMATOPOIETIC PROTEIN AND MATERIALS AND METHODS FOR MAKING IT**

## (57) Abstract

Hematopoietic proteins and polypeptide fragments thereof are provided, including proteins and polypeptides from mice and humans. Also provided are DNA molecules encoding the proteins and polypeptides, as well as vectors and cells useful in their production. Antibodies that bind to an epitope on the proteins are also provided. The proteins and polypeptides are useful for *in vivo* and *ex vivo* therapy, and as reagents for cell culture and investigation of cell proliferation and development.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Description

5 HEMATOPOIETIC PROTEIN AND MATERIALS AND METHODS FOR MAKING  
IT

Cross Reference to Related Application

10 This application is a continuation-in-part of  
Serial No. 08/215,203, filed March 21, 1994, which is a  
continuation-in-part of Serial No. 08/203,197, filed  
February 25, 1994, which is a continuation-in-part of  
Serial No. 08/196,025 filed February 14, 1994, which  
applications are pending and are incorporated herein by  
15 reference.

Background of the Invention

Hematopoiesis is the process by which blood  
cells develop and differentiate from pluripotent stem  
20 cells in the bone marrow. This process involves a complex  
interplay of polypeptide growth factors (cytokines) acting  
via membrane-bound receptors on the target cells.  
Cytokine action results in cellular proliferation and  
differentiation, with response to a particular cytokine  
25 often being lineage-specific and/or stage-specific.  
Development of a single cell type, such as a platelet,  
from a stem cell may require the coordinated action of a  
plurality of cytokines acting in the proper sequence.

The known cytokines include the interleukins,  
30 such as IL-1, IL-2, IL-3, IL-6, IL-8, etc.; and the colony  
stimulating factors, such as G-CSF, M-CSF, GM-CSF,  
erythropoietin (EPO), etc. In general, the interleukins  
act as mediators of immune and inflammatory responses.  
The colony stimulating factors stimulate the proliferation  
35 of marrow-derived cells, activate mature leukocytes, and  
otherwise form an integral part of the host's response to  
inflammatory, infectious, and immunologic challenges.

Various cytokines have been developed as therapeutic agents. For example, erythropoietin, which stimulates the development of erythrocytes, is used in the treatment of anemia arising from renal failure. Several  
5 of the colony stimulating factors have been used in conjunction with cancer chemotherapy to speed the recovery of patients' immune systems. Interleukin-2,  $\alpha$ -interferon and  $\gamma$ -interferon are used in the treatment of certain cancers. An activity that stimulates megakaryocytopoiesis  
10 and thrombocytopoiesis has been identified in body fluids of thrombocytopenic animals and is referred to in the literature as "thrombopoietin" (recently reviewed by McDonald, Exp. Hematol. 16:201-205, 1988 and McDonald, Am. J. Ped. Hematol. Oncol. 14:8-21, 1992). Despite more than  
15 three decades of study, the factor or factors responsible for this activity have not been definitively characterized, due in part to lack of a good source, a lack of good assays, and a lack of knowledge as to the site(s) of production.

20 Mild bleeding disorders (MBDs) associated with platelet dysfunctions are relatively common (Bachmann, Seminars in Hematology 17: 292-305, 1980), as are a number of congenital disorders of platelet function, including Bernard-Soulier syndrome (deficiency in platelet GPIb),  
25 Glanzmann's thrombasthenia (deficiency of GPIIb and GPIIIa), congenital afibrinogenemia (diminished or absent levels of fibrinogen in plasma and platelets), and gray platelet syndrome (absence of  $\alpha$ -granules). In addition there are a number of disorders associated with platelet  
30 secretion, storage pool deficiency, abnormalities in platelet arachidonic acid pathway, deficiencies of platelet cyclooxygenase and thromboxane synthetase and defects in platelet activation (reviewed by Rao and Holmsen, Seminars in Hematology 23: 102-118, 1986). At  
35 present, the molecular basis for most of these defects is not well understood.

The isolation and characterization of platelet proteins would provide invaluable tools for the elucidation of the underlying defects in many platelet dysfunctions. A major limiting step to detailed molecular analysis lies in difficulties in obtaining mRNA from platelets or from their precursor, the megakaryocyte, for analysis and cDNA library construction. Platelets are devoid of nuclei and transcription. The trace mRNAs still associated with platelets are difficult to isolate and are often subject to degradation. The construction of platelet cDNA libraries has heretofore required a large number of platelets, typically from 25 to 250 units of whole blood (Izumi et al., Proc. Natl. Acad. Sci. USA 87: 7477-7481, 1990; Wicki et al., Thrombosis and Haemostasis 61: 448-453, 1989; and Wenger et al., Blood 73: 1498-1503, 1989) or from pheresis of patients with elevated blood platelet counts due to essential thrombocythemia (Roth et al., Biochem. Biophys. Res. Comm. 160: 705-710, 1989). Where platelet-specific cDNAs have been isolated the mRNAs are probably the most stable or abundant of the total mRNA species and probably represent only a small fraction of the total coding repertoire of platelets.

An alternative route to a platelet cDNA library is the isolation and construction of a library from mRNA isolated from megakaryocytes, the direct cellular precursor to platelets. Megakaryocytes are polyploid cells and are expected to contain mRNA encoding the full complement of platelet and megakaryocytic proteins. However, it has proven difficult to isolate megakaryocytes in sufficient numbers and purity.

Recent advances in molecular biology have greatly increased our understanding of hematopoiesis, but at the same time have shown the process to be extremely complex. While many cytokines have been characterized and some have proven clinical applications, there remains a need in the art for additional agents that stimulate

proliferation and differentiation of myeloid and lymphoid precursors and the production of mature blood cells. There is a particular need for agents that stimulate the development and proliferation of cells of the megakaryocytic lineage, including platelets. There is a further need in the art for agents that can be used in the treatment of cytopenias, including thrombocytopenia, the condition of abnormally low number of circulating platelets (less than about  $1 \times 10^5$  platelets/ $\text{mm}^3$ ), and other platelet disorders. The present invention fulfills these needs and provides other, related advantages.

#### Summary of the Invention

It is an object of the present invention to provide isolated proteins having hematopoietic activity.

It is a further object of the invention to provide methods for producing proteins having hematopoietic activity, as well as isolated DNA molecules, vectors and cells that can be used within the methods.

It is a further object of the invention to provide antibodies that bind an epitope on a hematopoietic protein.

It is a further object of the invention to provide methods for stimulating the production of megakaryocytes, platelets and neutrophils in mammals including humans.

It is a further object of the invention to provide a variety of tools for use in the study of bone marrow cell development, differentiation and proliferation; and in the detection of diseases characterized by abnormalities in bone marrow cell development, differentiation and proliferation.

Within one aspect, the present invention provides an isolated protein selected from the group consisting of (a) proteins comprising the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to

amino acid residue 196; (b) proteins comprising the sequence of amino acids of SEQ ID NO: 2 from amino acid residue 45 to amino acid residue 206; (c) proteins comprising the sequence of amino acids of SEQ ID NO: 19 from amino acid residue 22 to amino acid residue 173; (d) proteins comprising the sequence of amino acids of SEQ ID NO: 19 from amino acid residue 22 to amino acid residue 175; (e) allelic variants of (a), (b), (c) and (d); and (f) species homologs of (a), (b), (c), (d) or (e) wherein the protein stimulates proliferation or differentiation of myeloid or lymphoid precursors. In certain embodiments, the protein comprises the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 379 or the sequence of amino acids of SEQ ID NO: 19 from amino acid residue 22 to amino acid residue 353.

Within a related aspect, the invention provides an isolated polynucleotide molecule encoding a protein as disclosed above. Within one embodiment, the polynucleotide molecule is a DNA molecule comprising a coding strand comprising the sequence of nucleotides of SEQ ID NO:1 from nucleotide 237 to nucleotide 692 or the sequence of nucleotides of SEQ ID NO: 18 from nucleotide 64 to nucleotide 519. Within other embodiments, the molecule comprises nucleotides 237-1241, 174-1241, 105-1241, 105-722, 174-722 or 237-722 of SEQ ID NO:1 or corresponding regions of SEQ ID NO: 18. The invention further provides allelic variants of these molecules and DNA molecules encoding a hematopoietic protein, which molecules encode a protein that is at least 80% identical in amino acid sequence to a protein encoded by one of the recited portions of SEQ ID NO:1 or SEQ ID NO:18. Molecules complementary to these sequences are also provided.

Within another aspect, the invention provides an isolated DNA molecule selected from the group consisting of (a) the Eco RI-Xho I insert of plasmid pZGmpl-1081

(ATCC 69566), (b) allelic variants of (a), and (c) DNA molecules encoding a protein that is at least 80% identical in amino acid sequence to a protein encoded by (a) or (b), wherein the isolated DNA molecule encodes a protein having hematopoietic activity.

Within another aspect, the invention provides an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment selected from the group consisting of (a) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 237 to nucleotide 692, (b) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:18 from nucleotide 64 to nucleotide 519; (c) allelic variants of (a) or (b), and (d) DNA segments encoding a hematopoietic protein that is at least 80% identical in amino acid sequence to a protein encoded by (a), (b) or (c); and a transcription terminator.

Within another aspect, the invention provides a cultured cell into which has been introduced an expression vector as disclosed above, wherein the cell expresses a hematopoietic protein encoded by the DNA segment. Within certain embodiments, the cell is a fungal cell, a mammalian cell or a bacterial cell.

Within another aspect, the invention provides a non-human mammal into the germ line of which has been introduced a heterologous DNA segment encoding a hematopoietic protein as disclosed above, wherein the mammal produces the hematopoietic protein encoded by said DNA segment.

Within another aspect, the invention provides methods for stimulating platelet production in a mammal. The methods comprise administering to a mammal a therapeutically effective amount of a hematopoietic protein selected from the group consisting of (a) proteins comprising the sequence of amino acids of SEQ ID



NO:2 from amino acid residue 45 to amino acid residue 196;  
(b) proteins comprising the sequence of amino acids of  
SEQ ID NO: 19 from amino acid residue 22 to amino acid  
residue 173; (c) allelic variants of (a) and (b); and (d)  
5 species homologs of (a), (b) or (c), wherein the protein  
stimulates proliferation or differentiation of myeloid or  
lymphoid precursors, in combination with a  
pharmaceutically acceptable vehicle.

These and other aspects of the invention will  
10 become evident upon reference to the following detailed  
description and the attached drawings.

#### Brief Description of the Drawings

Figure 1 is a partial restriction map of the  
15 vector pDX. Symbols used are SV40 ori, origin of  
replication from SV40; SV40 E, SV40 enhancer; MLP,  
adenovirus major late promoter; L1-3, adenovirus  
tripartite leader; ss, splicing signals; pA,  
polyadenylation site.

20 Figure 2 illustrates the construction of plasmid  
pBJ3. Symbols used are TPiP, TPi1 promoter; TPiT, TPi1  
terminator; AAT,  $\alpha$ -1 antitrypsin cDNA; alpha, alpha-factor  
leader; mTPO, mouse TPO coding sequence.

#### 25 Detailed Description of the Invention

Prior to describing the present invention in  
detail, it may be helpful to define certain terms used  
herein:

Allelic variant: An alternative form of a gene  
30 that arises through mutation, or an altered polypeptide  
encoded by the mutated gene. Gene mutations can be silent  
(no change in the encoded polypeptide) or may encode  
polypeptides having altered amino acid sequence.

cDNA: Complementary DNA, prepared by reverse  
35 transcription of a messenger RNA template, or a clone or

amplified copy of such a molecule. Complementary DNA can be single-stranded or double-stranded.

Expression vector: A DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both. The term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

Gene: A segment of chromosomal DNA that encodes a polypeptide chain. A gene includes one or more regions encoding amino acids, which in some cases are interspersed with non-coding "intervening sequences" ("introns"), together with flanking, non-coding regions which provide for transcription of the coding sequence.

Molecules complementary to: Polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

Promoter: The portion of a gene at which RNA polymerase binds and mRNA synthesis is initiated.

As noted above, the present invention provides materials and methods for use in producing proteins having hematopoietic activity. As used herein, the term "hematopoietic" denotes the ability to stimulate the proliferation and/or differentiation of myeloid or lymphoid precursors as determined by standard assays. See, for example, Metcalf, Proc. Natl. Acad. Sci. USA 77:

5327-5330, 1980; Metcalf et al., J. Cell. Physiol. 116: 198-206, 1983; and Metcalf et al., Exp. Hematol. 15: 288-295, 1987. Typically, marrow cells are incubated in the presence of a test sample and a control sample. The  
5 cultures are then scored for cell proliferation and differentiation by visual examination and/or staining. A particularly preferred assay is the MTT colorimetric assay of Mosman (J. Immunol. Meth. 65: 55-63, 1983; incorporated herein by reference) disclosed in more detail in the  
10 examples which follow.

The present invention is based in part upon the discovery of an activity that stimulates cell growth via the MPL receptor. This receptor (Souyri et al., Cell 63: 1137-1147, 1990) was, prior to this discovery, an "orphan"  
15 receptor whose natural ligand was unknown. Through processes of cloning and mutagenesis described in detail in the Examples which follow, the inventors developed a cell line that was dependent upon stimulation of an MPL receptor-linked pathway for its survival and growth, and  
20 which was capable of autocrine stimulation of the receptor. Conditioned media from these interleukin-3 (IL-3) independent cells was found to support the growth of cells that expressed the MPL receptor and were otherwise dependent on IL-3. Antibody neutralization experiments  
25 demonstrated that this activity was not due to IL-3 or IL-4, and that it could be neutralized by a soluble form of the MPL receptor. A cDNA library was then prepared from the IL-3 independent cell line. The DNA was used to transfect baby hamster kidney (BHK) cells, and media from  
30 the transfectants were assayed for the ability to stimulate MPL-dependent cell proliferation. A positive clone was isolated, and recombinant MPL ligand was produced. The recombinant protein was found to stimulate the proliferation of a broad spectrum of myeloid and  
35 lymphoid precursors, and, in particular, to stimulate production of megakaryocytes and neutrophils from

progenitor cells in the bone marrow. In addition, the recombinant protein was found to stimulate the production of platelets in test animals. In view of these activities, the protein has been designated thrombopoietin (TPO).

The present invention provides isolated polynucleotide molecules encoding thrombopoietin. Useful polynucleotide molecules in this regard include mRNA, genomic DNA, cDNA, synthetic DNA and DNA molecules generated by ligation of fragments from different sources. For production of recombinant TPO, DNA molecules lacking introns are preferred for use in most expression systems. By "isolated" it is meant that the molecules are removed from their natural genetic milieu. Thus, the invention provides DNA molecules free of other genes with which they are ordinarily associated. In particular, the molecules are free of extraneous or unwanted coding sequences, and in a form suitable for use within genetically engineered protein production systems.

The sequences of cDNA clones encoding representative mouse and human TPO proteins are shown in SEQ ID NO: 1 and SEQ ID NO:18, respectively, and the corresponding amino acid sequences are shown in SEQ ID NO: 2 and SEQ ID NO:19, respectively. Those skilled in the art will recognize that the sequences shown in SEQ ID NOS: 1, 2, 18 and 19, and the genomic sequences shown in SEQ ID NOS: 28 and 29, correspond to single alleles of the murine or human gene, and that allelic variation is expected to exist. Allelic variants of the DNA sequences shown in SEQ ID NO: 1, SEQ ID NO:18 and SEQ ID NO: 28, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO: 2 and SEQ ID NO:19. It will also be evident that one skilled in the art could

engineer sites that would facilitate manipulation of the nucleotide sequence using alternative codons.

The murine and human sequences disclosed herein are useful tools for preparing isolated polynucleotide molecules encoding TPO proteins from other species ("species homologs"). Preferred such species homologs include mammalian homologs such as bovine, canine, porcine, ovine, equine and, in particular, primate proteins. Methods for using sequence information from a first species to clone a corresponding polynucleotide sequence from a second species are well known in the art. See, for example, Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987. The DNA molecules of the present invention encoding TPO are generally at least 60%, preferably at least 80%, and may be 90-95% or more identical in sequence to SEQ ID NO: 1 and SEQ ID NO:18 and their allelic variants. Thrombopoietin molecules are characterized by their ability to specifically bind to MPL receptor from the same species and to stimulate platelet production in vivo. In normal test animals, TPO is able to increase platelet levels by 100% or more within 10 days after beginning daily administration.

Analysis of mRNA distribution showed that mRNA encoding TPO was present in several tissues of human and mouse, and was more abundant in lung, liver, heart, skeletal muscle and kidney. Thus, to isolate homologs from other species, a cDNA library is prepared, preferably from one of the tissues found to produce higher levels of the mRNA. Methods for preparing cDNA libraries are well known in the art. See, for example, Sambrook et al., eds., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989 and references cited therein. To detect molecules encoding TPO, the library is then probed with the mouse or human cDNA disclosed herein or with a fragment thereof or with one or

more small probes based on the disclosed sequences. Of particular utility are probes comprising an oligonucleotide of at least about 14 or more nucleotides and up to 25 or more nucleotides in length that are at least 80% identical to a same-length portion of SEQ ID NO: 1, SEQ ID NO: 18, SEQ ID NO: 28 or their complementary sequences. It is preferred to probe the library at a low hybridization stringency, i.e. about 2x SSC and a hybridization temperature of about 50°C using labeled probes. Molecules to which the probe hybridizes are detected using standard detection procedures. Positive clones are confirmed by sequence analysis and activity assays, such as ability to bind homologous MPL receptor (i.e. an MPL receptor from the same species as the cDNA) or to stimulate hematopoiesis from homologous marrow cells. As will be evident to one skilled in the art, other cloning methods can be utilized.

Polynucleotide molecules encoding TPO (including allelic variants and species homologs of the molecules disclosed herein) can also be isolated by cloning from a cell line that produces the MPL ligand and exhibits autocrine growth stimulation. Briefly, a factor-dependent cell line is transfected to express an MPL receptor (Vigon et al., Proc. Natl. Acad. Sci. USA 89: 5640-5644, 1992; Skoda et al., EMBO J. 12: 2645-2653, 1993; and SEQ ID NO: 17), then mutagenized, and factor-independent cells are selected. These cells are then used as a source of TPO mRNA. Suitable factor-dependent cell lines include the IL-3-dependent BaF3 cell line (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol. 6: 4133-4135, 1986), FDC-P1 (Hapel et al., Blood 64: 786-790, 1984), and MO7e (Kiss et al., Leukemia 7: 235-240, 1993). Growth factor-dependent cell lines can be established according to published methods (e.g. Greenberger et al., Leukemia Res. 8: 363-375, 1984; Dexter et al., in Baum et al. Eds., Experimental Hematology

Today, 8th Ann. Mtg. Int. Soc. Exp. Hematol. 1979, 145-156, 1980). In a typical procedure, cells are removed from the tissue of interest (e.g. bone marrow, spleen, fetal liver) and cultured in a conventional, serum-supplemented medium, such as RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 15% horse serum and  $10^{-6}$  M hydrocortisone. At one- to two-week intervals non-adherent cells are harvested, and the cultures are fed fresh medium. The harvested, non-adherent cells are washed and cultured in medium with an added source of growth factor (e.g. RPMI 1640 + 10% FBS + 5-20% WEHI-3 conditioned medium as a source of IL-3). These cells are fed fresh medium at one- to two-week intervals and expanded as the culture grows. After several weeks to several months, individual clones are isolated by plating the cells onto semi-solid medium (e.g. medium containing methylcellulose) or by limiting dilution. Factor dependence of the clones is confirmed by culturing individual clones in the absence of the growth factor. Retroviral infection or chemical mutagenesis can be used to obtain a higher frequency of growth factor-dependent cells. The factor-dependent cells are transfected to express the MPL receptor, then mutagenized, such as by chemical treatment, exposure to ultraviolet light, exposure to x-rays, or retroviral insertional mutagenesis. The mutagenized cells are then cultured under conditions in which cell survival is dependent upon autocrine growth factor production, that is in the absence of the exogenous growth factor(s) required by the parent cell. Production of TPO is confirmed by screening, such as by testing conditioned media on cells expressing and not expressing MPL receptor or by testing the activity of conditioned media in the presence of soluble MPL receptor or antibodies against known cytokines.

The present invention also provides isolated proteins that are substantially homologous to the proteins

of SEQ ID NO: 2 or SEQ ID NO:19 and their species homologs. By "isolated" is meant a protein which is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the proteins in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. The term "substantially homologous" is used herein to denote proteins having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequences shown in SEQ ID NO: 2 or SEQ ID NO:19 or their species homologs. Such proteins will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO: 2 or SEQ ID NO:19 or their species homologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-616, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 1 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$



Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-2	-1	1	5				
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Substantially homologous proteins are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 2); small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference.

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

Essential amino acids in TPO may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. receptor binding, *in vitro* or *in vivo* proliferative activity) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., Science 255:306-312, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992.

In general, cytokines are predicted to have a four-alpha helix structure, with the first and fourth helices being most important in ligand-receptor interactions and more highly conserved among members of the family. Referring to the human TPO amino acid sequence shown in SEQ ID NO:19, alignment of cytokine sequences suggests that these helices are bounded by amino acid residues 29 and 53, 80 and 99, 108 and 130, and 144 and 168, respectively (boundaries are  $\pm 4$  residues). Helix boundaries of the mouse (SEQ ID NO:2) and other non-human TPOs can be determined by alignment with the human sequence. Other important structural aspects of TPO include the cysteine residues at positions 51, 73, 129 and 195 of SEQ ID NO:2 (corresponding to positions 28, 50, 106 and 172 of SEQ ID NO:19).

In addition, the proteins of the present invention (or polypeptide fragments thereof) can be joined to other bioactive molecules, particularly other cytokines, to provide multi-functional molecules. For

example, the C-terminal domain of thrombopoietin can be joined to other cytokines to enhance their biological properties or efficiency of production. The thrombopoietin molecule appears to be composed of two domains. The first (amino-terminal) domain of approximately 150 amino acids is similar in size and bears structural resemblance to erythropoietin and several other hematopoietic cytokines. Following this first domain is a second domain of approximately 180 amino acids, which has a structure that is not significantly similar to any known protein structure in databases. This second domain is highly enriched in N-linked glycosylation sites and in serine, proline, and threonine residues, which are hallmarks of O-linked glycosylation sites. This apparently high carbohydrate content suggests that this domain plays a role in making the hydrophobic first domain relatively more soluble. Experimental evidence indicates that the carbohydrate associated with the second domain is involved in proper intracellular assembly and secretion of the protein during its biosynthesis. The second domain may also play a role in stabilizing the first domain against proteolytic degradation and/or prolonging the *in vivo* half-life of the molecule, and may potentiate biological signal transmittance or specific activity of the protein.

The present invention thus provides a series of novel, hybrid molecules in which the second domain of TPO is joined to a second cytokine. It is preferred to join the C-terminal domain of TPO to the C-terminus of the second cytokine. Joining is preferably done by splicing at the DNA level to allow expression of chimeric molecules in recombinant production systems. The resultant molecules are then assayed for such properties as improved solubility, improved stability, prolonged clearance half-life, or improved expression and secretion levels, and pharmacodynamics. Specific examples of such chimeric

cytokines include those in which the second domain of TPO is joined to the C-terminus of EPO, G-CSF, GM-CSF, IL-6, IL-3, or IL-11. As noted above, this is conveniently done by DNA fusion. The fused cDNA is then subcloned into a suitable expression vector and transformed or transfected into host cells or organisms according to conventional methods. The resulting fusion proteins are purified using conventional chromatographic purification techniques (e.g. chromatographic techniques), and their properties are compared with those of the native, non-fused, parent cytokine. Such hybrid molecules may further comprise additional amino acid residues (e.g. a polypeptide linker) between the component proteins or polypeptides.

In addition to the hematopoietic proteins disclosed above, the present invention includes fragments of these proteins and isolated polynucleotide molecules encoding the fragments. Of particular interest are fragments of at least 10 amino acids in length that bind to an MPL receptor, and polynucleotide molecules of at least 30 nucleotides in length encoding such polypeptides. Polypeptides of this type are identified by known screening methods, such as by digesting the intact protein or synthesizing small, overlapping polypeptides or polynucleotides (and expressing the latter), optionally in combination with the techniques of structural analysis disclosed above. The resultant polypeptides are then tested for the ability to specifically bind the MPL receptor and stimulate cell proliferation via the MPL receptor. Binding is determined by conventional methods, such as that disclosed by Klotz, Science 217: 1247, 1982 ("Scatchard analysis"). Briefly, a radiolabeled test polypeptide is incubated with MPL receptor-bearing cells in the presence of increasing concentrations of unlabeled TPO. Cell-bound, labeled polypeptide is separated from free labeled polypeptide by centrifugation through phthalate oil. The binding affinity of the test

polypeptide is determined by plotting the ratio of bound to free label on the ordinate versus bound label on the abscissa. Binding specificity is determined by competition with cytokines other than TPO. Receptor binding can also be determined by precipitation of the test compound by immobilized MPL receptor (or the ligand-binding extracellular domain thereof). Briefly, the receptor or portion thereof is immobilized on an insoluble support. The test compound is labeled, e.g. by metabolically labeling of the host cells in the case of a recombinant test compound, or by conventional, *in vitro* labeling methods (e.g. radio-iodination). The labeled compound is then combined with the immobilized receptor, unbound material is removed, and bound, labeled compound is detected. Methods for detecting a variety of labels are known in the art. Stimulation of proliferation is conveniently determined using the MTT colorimetric assay with MPL receptor-bearing cells. Polypeptides are assayed for activity at various concentrations, typically over a range of 1 nM to 1 mM.

Larger polypeptides of up to 50 or more residues, preferably 100 or more residues, more preferably about 140 or more residues, up to the size of the entire mature protein are also provided. For example, analysis and modeling of the amino acid sequence shown in SEQ ID NO: 2 from residue 51 to residue 195, inclusive, or SEQ ID NO: 19 from residue 28 to residue 172, inclusive, suggest that these portions of the molecules are cytokine-like domains capable of self assembly. Also of interest are molecules containing this core cytokine-like domain plus one or more additional segments or domains of the primary translation product. Thus, other polypeptides of interest include those shown in Table 3.

Table 3

## Mouse TPO (SEQ ID NO:2):

	Cys (residue 51)--Val (residue 196)
5	Cys (51)--Pro (206)
	Cys (51)--Thr (379)
	Ser (45)--Cys (195)
	Ser (45)--Val (196)
	Ser (45)--Pro (206)
10	Ser (45)--Thr (379)
	Met (24)--Cys (195)
	Met (24)--Val (196)
	Met (24)--Pro (206)
	Met (24)--Thr (379)
15	Met (1)--Cys (195)
	Met (1)--Val (196)
	Met (1)--Pro (206)
	Met (1)--Thr (379)

## Human TPO (SEQ ID NO:19)

20	Cys (28)--Val (173)
	Cys (28)--Arg (175)
	Cys (28)--Gly (353)
	Ser (22)--Cys (172)
	Ser (22)--Val (173)
25	Ser (22)--Arg (175)
	Ser (22)--Gly (353)
	Met (1)--Cys (172)
	Met (1)--Val (173)
	Met (1)--Arg (175)
30	Met (1)--Gly (353)

Those skilled in the art will recognize that intermediate forms of the molecules (e.g those having C-termini between residues 196 and 206 of SEQ ID NO:2 or  
 35 those having N-termini between residues 22 and 28 of SEQ

ID NO:19) are also of interest, as are polypeptides having one or more amino acid substitutions, deletions, insertions, or N- or C-terminal extensions as disclosed above. Thus, the present invention provides hematopoietic polypeptides of at least 10 amino acid residues, preferably at least 50 residues, more preferably at least 100 residues and most preferably at least about 140 residues in length, wherein said polypeptides are substantially homologous to like-size polypeptides of SEQ ID NO:2 or SEQ ID NO:19.

The proteins of the present invention can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., *ibid.*, which are incorporated herein by reference.

In general, a DNA sequence encoding a protein of the present invention is operably linked to a transcription promoter and terminator within an expression vector. The vector will commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.



To direct a protein of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence is joined to the DNA sequence encoding a protein of the present invention in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the protein of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). The secretory signal sequence may be that normally associated with a protein of the present invention, or may be from a gene encoding another secreted protein.

Yeast cells, particularly cells of the genus *Saccharomyces*, are a preferred host for use within the present invention. Methods for transforming yeast cells with exogenous DNA and producing recombinant proteins therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075, which are incorporated herein by reference. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g. leucine). A preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. A preferred secretory signal sequence for use in yeast is that of the *S. cerevisiae* *MF $\alpha$ 1* gene (Brake, *ibid.*; Kurjan et al., U.S. Patent No. 4,546,082). Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S.

Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092, which are incorporated herein by reference) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 5 4,990,446; 5,063,154; 5,139,936 and 4,661,454, which are incorporated herein by reference. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, 10 *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-3465, 1986 and Cregg, U.S. Patent No. 4,882,279.

Other fungal cells are also suitable as host cells. For example, *Aspergillus* cells may be utilized 15 according to the methods of McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228, which is incorporated herein by reference. Methods for 20 transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533, which is incorporated herein by reference.

Cultured mammalian cells are also preferred hosts within the present invention. Methods for 25 introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 30 1:841-845, 1982) and DEAE-dextran mediated transfection (Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), which are incorporated herein by reference. The production of recombinant proteins in cultured mammalian cells is 35 disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950;

Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference. Preferred cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651),  
5 BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories  
10 such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from  
15 metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978, which are incorporated herein by reference) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been  
20 inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is  
25 a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as  
30 "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A  
35 preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate.

Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used  
5 as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign proteins therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463, which are  
10 incorporated herein by reference. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987.

Preferred prokaryotic host cells for use in  
15 carrying out the present invention are strains of the bacteria *Escherichia coli*, although *Bacillus* and other genera are also useful. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al.,  
20 *ibid.*). When expressing the proteins in bacteria such as *E. coli*, the protein may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are  
25 recovered and denatured using, for example, guanidine isothiocyanate. The denatured protein is then refolded by diluting the denaturant. In the latter case, the protein can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for  
30 example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein.

Transformed or transfected host cells are  
cultured according to conventional procedures in a culture  
35 medium containing nutrients and other components required for the growth of the chosen host cells. A variety of

suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

Within the present invention, transgenic animal technology may be employed to produce TPO. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically. Furthermore, the major milk proteins are present in milk at high concentrations (from about 1 to 15 g/l).

From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof-of-concept stage), within the present invention it is preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk. See WIPO Publication WO 88/00239 for a comparison of factors influencing the choice of host species. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic

suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

Within the present invention, transgenic animal technology may be employed to produce TPO. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically. Furthermore, the major milk proteins are present in milk at high concentrations (from about 1 to 15 g/l).

From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof-of-concept stage), within the present invention it is preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of equipment for collecting sheep milk. See WIPO Publication WO 88/00239 for a comparison of factors influencing the choice of host species. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic

line at a later date. In any event, animals of known, good health status should be used.

To obtain expression in the mammary gland, a transcription promoter from a milk protein gene is used.

5 Milk protein genes include those genes encoding caseins (see U.S. Patent No. 5,304,489, incorporated herein by reference), beta-lactoglobulin,  $\alpha$ -lactalbumin, and whey acidic protein. The beta-lactoglobulin (BLG) promoter is preferred. In the case of the ovine beta-lactoglobulin

10 gene, a region of at least the proximal 406 bp of 5' flanking sequence of the gene will generally be used, although larger portions of the 5' flanking sequence, up to about 5 kbp, are preferred, such as a -4.25 kbp DNA segment encompassing the 5' flanking promoter and non-

15 coding portion of the beta-lactoglobulin gene. See Whitelaw et al., Biochem J. 286: 31-39, 1992. Similar fragments of promoter DNA from other species are also suitable.

Other regions of the beta-lactoglobulin gene may

20 also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in comparison with those that contain such DNA sequences (see Brinster et al., Proc. Natl. Acad. Sci. USA 85: 836-840, 1988; Palmiter et al., Proc. Natl. Acad. Sci. USA 88: 478-482, 1991; Whitelaw et al., Transgenic Res. 1: 3-13, 1991; WO 89/01343; WO 91/02318). In this regard, it is generally preferred, where possible, to use

30 genomic sequences containing all or some of the native introns of a gene encoding the protein or polypeptide of interest, thus the further inclusion of at least some introns from, e.g., the beta-lactoglobulin gene, is preferred. One such region is a DNA segment which provides for intron splicing and RNA polyadenylation from

35 the 3' non-coding region of the ovine beta-lactoglobulin gene. When substituted for the natural 3' non-coding

sequences of a gene, this ovine beta-lactoglobulin segment can both enhance and stabilize expression levels of the protein or polypeptide of interest. Within other embodiments, the region surrounding the initiation ATG of the TPO sequence is replaced with corresponding sequences from a milk specific protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression. It is convenient to replace the entire TPO pre-pro and 5' non-coding sequences with those of, for example, the BLG gene, although smaller regions may be replaced.

For expression of TPO in transgenic animals, a DNA segment encoding TPO is operably linked to additional DNA segments required for its expression to produce expression units. Such additional segments include the above-mentioned promoter, as well as sequences which provide for termination of transcription and polyadenylation of mRNA. The expression units will further include a DNA segment encoding a secretory signal sequence operably linked to the segment encoding TPO. The secretory signal sequence may be a native TPO secretory signal sequence or may be that of another protein, such as a milk protein. See, for example, von Heinje, Nuc. Acids Res. 14: 4683-4690, 1986; and Meade et al., U.S. Patent No. 4,873,316, which are incorporated herein by reference.

Construction of expression units for use in transgenic animals is conveniently carried out by inserting a TPO sequence into a plasmid or phage vector containing the additional DNA segments, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA segment encoding a milk protein and to replace the coding sequence for the milk protein with that of a TPO polypeptide, thereby creating a gene fusion that includes the expression control sequences of the milk protein gene. In any event, cloning of the



expression units in plasmids or other vectors facilitates the amplification of the TPO sequence. Amplification is conveniently carried out in bacterial (e.g. *E. coli*) host cells, thus the vectors will typically include an origin of replication and a selectable marker functional in bacterial host cells.

The expression unit is then introduced into fertilized eggs (including early-stage embryos) of the chosen host species. Introduction of heterologous DNA can be accomplished by one of several routes, including microinjection (e.g. U.S. Patent No. 4,873,191), retroviral infection (Jaenisch, Science 240: 1468-1474, 1988) or site-directed integration using embryonic stem (ES) cells (reviewed by Bradley et al., Bio/Technology 10: 534-539, 1992). The eggs are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop to term. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny in the normal, Mendelian fashion, allowing the development of transgenic herds.

General procedures for producing transgenic animals are known in the art. See, for example, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986; Simons et al., Bio/Technology 6: 179-183, 1988; Wall et al., Biol. Reprod. 32: 645-651, 1985; Buhler et al., Bio/Technology 8: 140-143, 1990; Ebert et al., Bio/Technology 9: 835-838, 1991; Krimpenfort et al., Bio/Technology 9: 844-847, 1991; Wall et al., J. Cell. Biochem. 49: 113-120, 1992; U.S. Patents Nos. 4,873,191 and 4,873,316; WIPO publications WO 88/00239, WO 90/05188, WO 92/11757; and GB 87/00458, which are incorporated herein by reference. Techniques for introducing foreign DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g., Gordon et al., Proc. Natl. Acad. Sci. USA 77: 7380-7384, 1980; Gordon and Ruddle, Science 214: 1244-1246,

1981; Palmiter and Brinster, Cell 41: 343-345, 1985; Brinster et al., Proc. Natl. Acad. Sci. USA 82: 4438-4442, 1985; and Hogan et al. (ibid.). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WIPO publications WO 88/00239, WO 90/05188, and WO 92/11757; and Simons et al., Bio/Technology 6: 179-183, 1988). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to techniques which have become standard in the art. Injection of DNA into the cytoplasm of a zygote can also be employed.

Production in transgenic plants may also be employed. Expression may be generalized or directed to a particular organ, such as a tuber. See, Hiatt, Nature 344:469-479, 1990; Edelbaum et al., J. Interferon Res. 12:449-453, 1992; Sijmons et al., Bio/Technology 8:217-221, 1990; and European Patent Office Publication EP 255,378.

TPO prepared according to the present invention is purified using methods generally known in the art, such as affinity purification and separations based on size, charge, solubility and other properties of the protein. When the protein is produced in cultured mammalian cells, it is preferred to culture the cells in a serum-free culture medium in order to limit the amount of contaminating protein. The medium is harvested and fractionated. Preferred methods of fractionation include affinity chromatography on concanavalin A or other lectin, thereby making use of the carbohydrate present on the protein. The proteins can also be purified using an immobilized MPL receptor protein or ligand-binding portion thereof or through the use of an affinity tag (e.g. polyhistidine, substance P or other polypeptide or protein for which an antibody or other specific binding agent is

available). A specific cleavage site may be provided between the protein of interest and the affinity tag.

The proteins of the present invention can be used therapeutically wherever it is desirable to increase proliferation of cells in the bone marrow, such as in the treatment of cytopenia, such as that induced by aplastic anemia, myelodysplastic syndromes, chemotherapy or congenital cytopenias; in bone marrow transplant patients; in peripheral blood stem cell transplant patients; and in the treatment of conditions that cause bone marrow failure, such as myelodysplastic syndrome. The proteins are also useful for increasing platelet production, such as in the treatment of thrombocytopenia. Thrombocytopenia is associated with a diverse group of diseases and clinical situations that may act alone or in concert to produce the condition. Lowered platelet counts can result from, for example, defects in platelet production (due to, e.g., congenital disorders such as Fanconi syndrome, thrombocytopenia absent radii syndrome, Wiskott Aldrich, May Hegglin anomaly, Bernard-Soulier syndrome, Menneapolis syndrome, Epstein syndrome, Montreal platelet syndrome and Eckstein syndrome), abnormal platelet distribution, dilutional losses due to massive transfusions, abnormal destruction of platelets, or abnormal sequestration of platelets in the spleens of hypersplenic patients (due to, e.g., cirrhosis or congestive heart failure). For example, chemotherapeutic drugs used in cancer therapy may suppress development of platelet progenitor cells in the bone marrow, and the resulting thrombocytopenia limits the chemotherapy and may necessitate transfusions. In addition, certain malignancies can impair platelet production and platelet distribution. Radiation therapy used to kill malignant cells also kills platelet progenitor cells. Thrombocytopenia may also arise from various platelet autoimmune disorders induced by drugs, neonatal alloimmunity, platelet transfusion alloimmunity

and viral (including HIV) infection. The proteins of the present invention can reduce or eliminate the need for transfusions, thereby reducing the incidence of platelet alloimmunity. Abnormal destruction of platelets can

5 result from: (1) increased platelet consumption in vascular grafts or traumatized tissue; or (2) immune mechanisms associated with, for example, drug-induced thrombocytopenia, idiopathic thrombocytopenic purpura (ITP), autoimmune diseases, hematologic disorders such as  
10 leukemia and lymphoma or metastatic cancers involving bone marrow. Other indications for the proteins of the present invention include aplastic anemia and drug-induced marrow suppression resulting from, for example, chemotherapy or treatment of HIV infection with AZT.

15 Thrombocytopenia is manifested as increased bleeding, such as mucosal bleedings from the nasal-oral area or the gastrointestinal tract, as well as oozing from wounds, ulcers or injection sites.

For pharmaceutical use, the proteins of the  
20 present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general,  
25 pharmaceutical formulations will include a hematopoietic protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering  
30 agents, albumin to prevent protein loss on vial surfaces, etc. In addition, the hematopoietic proteins of the present invention may be combined with other cytokines, particularly early-acting cytokines such as stem cell factor, IL-3, IL-6, IL-11 or GM-CSF. When utilizing such  
35 a combination therapy, the cytokines may be combined in a single formulation or may be administered in separate

formulations. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, Gennaro, ed., Mack Publishing Co., Easton PA, 1990, which is incorporated herein by reference. Therapeutic doses will generally be in the range of 0.1 to 100  $\mu\text{g/kg}$  of patient weight per day, preferably 0.5-20  $\mu\text{g/kg}$  per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins will commonly be administered over a period of up to 28 days following chemotherapy or bone-marrow transplant or until a platelet count of  $>20,000/\text{mm}^3$ , preferably  $>50,000/\text{mm}^3$ , is achieved. More commonly, the proteins will be administered over one week or less, often over a period of one to three days. In general, a therapeutically effective amount of TPO is an amount sufficient to produce a clinically significant increase in the proliferation and/or differentiation of lymphoid or myeloid progenitor cells, which will be manifested as an increase in circulating levels of mature cells (e.g. platelets or neutrophils). Treatment of platelet disorders will thus be continued until a platelet count of at least  $20,000/\text{mm}^3$ , preferably  $50,000/\text{mm}^3$ , is reached. The proteins of the present invention can also be administered in combination with other cytokines such as IL-3, -6 and -11; stem cell factor; erythropoietin; G-CSF and GM-CSF. Within regimens of combination therapy, daily doses of other cytokines will in general be: EPO,  $\leq 150 \text{ U/kg}$ ; GM-CSF, 5-15  $\mu\text{g/kg}$ ; IL-3, 1-5  $\mu\text{g/kg}$ ; and G-CSF, 1-25  $\mu\text{g/kg}$ . Combination therapy with EPO, for example, is indicated in anemic patients with low EPO levels.

The proteins of the present invention are also valuable tools for the *in vitro* study of the differentiation and development of hematopoietic cells,

such as for elucidating the mechanisms of cell differentiation and for determining the lineages of mature cells, and may also find utility as proliferative agents in cell culture.

5           The proteins of the present invention can also be used *ex vivo*, such as in autologous marrow culture. Briefly, bone marrow is removed from a patient prior to chemotherapy and treated with TPO, optionally in combination with one or more other cytokines. The treated  
10 marrow is then returned to the patient after chemotherapy to speed the recovery of the marrow. In addition, the proteins of the present invention can also be used for the *ex vivo* expansion of marrow or peripheral blood progenitor (PBPC) cells. Prior to chemotherapy treatment, marrow can  
15 be stimulated with stem cell factor (SCF) or G-CSF to release early progenitor cells into peripheral circulation. These progenitors can be collected and concentrated from peripheral blood and then treated in culture with TPO, optionally in combination with one or  
20 more other cytokines, including but not limited to SCF, G-CSF, IL-3, GM-CSF, IL-6 or IL-11, to differentiate and proliferate into high-density megakaryocyte cultures, which can then be returned to the patient following high-dose chemotherapy.

25           Antibodies that bind an epitope on a protein of the present invention are also provided. Such antibodies can be produced by a variety of means known in the art. The production of non-human, monoclonal antibodies is well known and may be accomplished by, for example, immunizing  
30 an animal such as a mouse, rat, rabbit, goat, sheep or guinea pig with a recombinant or synthetic TPO or a selected polypeptide fragment thereof. It is preferred to immunize the animal with a highly purified protein or polypeptide fragment. It is also preferred to administer  
35 the protein or polypeptide in combination with an adjuvant, such as Freund's adjuvant, in order to enhance

the immune response. Although a single injection of antigen may be sufficient to induce antibody production in the animal, it is generally preferred to administer a large initial injection followed by one or more booster  
5 injections over a period of several weeks to several months. See, e.g., Hurrell, ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boca Raton, FL, 1982, which is incorporated herein by reference. Blood is then collected from the animal and  
10 clotted, and antibodies are isolated from the serum using conventional techniques such as salt precipitation, ion exchange chromatography, affinity chromatography or high performance liquid chromatography.

The use of monoclonal antibodies is generally  
15 preferred over polyclonal antisera. Monoclonal antibodies provide the advantages of ease of production, specificity and reproducibility. Methods for producing monoclonal antibodies are well known in the art and are disclosed, for example, by Kohler and Milstein (Nature 256:495, 1975  
20 and Eur. J. Immunol. 6:511-519, 1976). See also Hurrell, *ibid.* and Hart, U.S. Patent No. 5,094,941, which are incorporated herein by reference. Briefly, antibody-producing cells obtained from immunized animals are immortalized and screened, or screened first, for the  
25 production of antibody that binds to TPO. Positive cells are then immortalized by fusion with myeloma cells. Non-human antibodies can be "humanized" according to known techniques. See, for example, U.S. Patent No. 4,816,397; European Patent Office Publications 173,494 and 239,400;  
30 and WIPO publications WO 87/02671 and WO 90/00616, which are incorporated herein by reference. Briefly, human constant region genes are joined to appropriate human or non-human variable region genes. For example, the amino acid sequences which represent the antigen binding sites  
35 (CDRs, or complementarity-determining regions) of the parent (non-human) monoclonal antibody are grafted at the

DNA level onto human variable region framework sequences. Methods for this technique are known in the art and are disclosed, for example, by Jones et al. (Nature 326: 522-525, 1986), Riechmann et al. (Nature 322: 323-327, 1988) and Queen et al. (Proc. Natl. Acad. Sci. USA 86: 10029-10033, 1989). The joined genes are then transfected into host cells, which are cultured according to conventional procedures. In the alternative, monoclonal antibody producing cells may be transfected with cloned human constant region genes, and chimeric antibody genes generated by homologous recombination. Thus it is possible to assemble monoclonal antibodies with a significant portion of the structure being human, thereby providing antibodies that are more suitable for multiple administrations to human patients.

Single chain antibodies can be developed through the expression of a recombinant polypeptide which is generally composed of a variable light-chain sequence joined, typically via a linker polypeptide, to a variable heavy-chain sequence. Methods for producing single chain antibodies are known in the art and are disclosed, for example, by Davis et al. (BioTechnology 9: 165-169, 1991).

Antibodies that bind to epitopes of TPO are useful, for example, in the diagnosis of diseases characterized by reduced levels of platelets, megakaryocytes or other blood or progenitor cells, which diseases are related to deficiencies in the proliferation or differentiation of progenitor cells. Such diagnosis will generally be carried out by testing blood or plasma using conventional immunoassay methods such as enzyme-linked immunoadsorption assays or radioimmune assays. Assays of these types are well known in the art. See, for example, Hart et al., Biochem. 29: 166-172, 1990; Ma et al., British Journal of Haematology 80: 431-436, 1992; and Andre et al., Clin. Chem. 38/5: 758-763, 1992. Diagnostic assays for TPO activity may be useful for identifying



patient populations most likely to benefit from TPO therapy. Antibodies to TPO are also useful in purification of TPO, such as by attaching an antibody to a solid support, such as a particulate matrix packed into a column, and passing a solution containing the protein over the column. Bound protein is then eluted with an appropriate buffer. In general, protein is bound to the column under physiological conditions of low ionic strength and near-neutral pH. The column is then washed to elute unbound contaminants. Elution of bound protein is carried out by changing ionic strength or pH, such as with 3M KSCN (batch or gradient) or low pH citrate buffer. A pH below about 2.5 should generally be avoided.

The present invention also provides methods for producing large numbers of megakaryocytes and platelets, which can be used, for example, for preparing cDNA libraries. Because platelets are directed to sites of injuries, they are believed to be mediators of wound healing and, under some circumstances, mediators of pathogenesis. Hence, a detailed understanding of platelet and megakaryocyte molecular biology would provide insights into both homeostasis and clinically relevant disorders of platelet functions. The proteins of the present invention provide an improved means for producing megakaryocyte or platelet cDNA libraries.

Recombinant thrombopoietin when administered to animals or applied to cultured spleen or bone marrow cells induces proliferation of megakaryocytes from precursor cells. The expansion of megakaryocytes and their precursors and megakaryocyte maturation following the administration of TPO enables isolation of megakaryocytes in high purity and sufficient number for mRNA isolation and cDNA library construction. By adjusting the TPO dosage and the administration regime, early or fully matured megakaryocytes and those which are actively shedding platelets can be selectively expanded from

primary spleen or bone marrow cells. Accordingly, representative cDNA libraries can be constructed corresponding to early, intermediate or late stages or megakaryopoiesis.

5           The uses of the resulting cDNA libraries are many. Such libraries can be used, for example, for the identification and cloning of low abundance proteins that play a role in various platelet dysfunctions. The ease with which patients' megakaryocytes can be expanded and  
10       their mRNA isolated for analysis greatly aids the molecular dissection of diseases. The libraries are also a source for the cloning of novel growth factors and other proteins with potential therapeutic utility. Useful platelet proteins already cloned include platelet derived  
15       growth factor (Ross et al., Cell 26: 155-169, 1986); transforming growth factor (Miletich et al., Blood 54: 1015-1023, 1979; Roberts and Sporn, Growth Factors 8: 1-9, 1993); platelet-derived endothelial cell growth factor (Miletich et al., Blood 54: 1015-1023, 1979) and PF-4 (Doi  
20       et al., Mol. Cell. Biol. 7: 898-904, 1987; Poncz et al., Blood 69: 219-223, 1987). Novel growth factors may be identified by functional screening of expression cDNA libraries or by hybridization screening at reduced stringency with known growth factor probes. The isolation  
25       of novel growth factors may also be done by polymerase chain reaction utilizing degenerate primers to conserved regions of known growth factors. In addition, the systematic and complete DNA sequencing of a library provides a megakaryocyte cDNA sequence data base. Such a  
30       data base can be mined for useful sequences by a variety of computer-based search algorithms.

          Megakaryocytes prepared as disclosed above can also be used to prepare a protein library. This protein library is complementary to the cDNA library. Amino acid  
35       sequence information obtained from the protein library enables rapid isolation of cDNAs encoding proteins of

interest. The use of protein sequence data to design primers for DNA isolation eliminates problems arising in conventional library preparation methods due to relative mRNA abundance. Coupling of protein and cDNA libraries  
5 also facilitates the targeted cloning of sequences of particular interest.

A protein library is prepared by extracting proteins (total proteins or fractions of interest) from megakaryocytes according to known methods, then separating  
10 the proteins by two-dimensional gel electrophoresis. Isolated proteins are then subjected to *in situ* tryptic digestion followed by separation by micro-bore HPLC. The separated fragments are then analyzed by mass spectrometry. The resulting mass profile is searched  
15 against a protein sequence data base to infer protein identity. Unidentified peptides can be sequenced by Edman degradation.

The cDNA and protein libraries are valuable sources of new proteins and the sequences encoding them.  
20 Platelets are believed to be important mediators of wound healing and, under some circumstances, pathogenesis. Many important platelet proteins have been identified and characterized, including platelet-derived growth factor, transforming growth factor- $\beta$ , platelet-derived endothelial  
25 cell growth factor, and platelet factor 4. Identification and characterization of other platelet proteins would be extremely helpful in the elucidation of the processes underlying wound healing and pathogenesis, and would be expected to yield important therapeutic agents and  
30 strategies.

As disclosed in more detail below, the human TPO gene has been localized to chromosome 3q26. This information, coupled with the sequence of the human TPO gene (SEQ ID NO:28), permits the direct diagnosis, by  
35 genetic screening, of inherited disorders in the TPO gene or the regulation of its expression. Such disorders may

include alterations in promoter sequences leading to increases or decreases in expression level, chromosomal translocations at coding or non-coding regions, and the juxtaposition of new regulatory sequences at the TPO locus. Diagnostic methods that can be applied are known in the art. For example, primers or hybridization probes of at least 5 nucleotides, preferably 15-30 or more nucleotides in length, can be designed from the genomic sequence and used to detect chromosomal abnormalities or measure mRNA levels. A variety of suitable detection and measurement methods are known in the art, and include "Southern" blotting, polymerase chain reaction (Mullis, U.S. Patent No. 4,683,202), and ligase chain reaction (Barany, PCR Methods and Applications 1:5-16, Cold Spring Harbor Laboratory Press, 1991). For example, patient DNA can be digested with one or more restriction enzymes and transferred to nitrocellulose to produce a Southern blot. The blot is then probed to detect gross changes in fragment sizes resulting from mutation in a restriction site recognition sequence. In another procedure, analysis of abnormal gene sequences and comparison of the normal and abnormal sequences allows the design of primers that can be used to identify the abnormal (e.g. disrupted or translocated) gene. Patient DNA is amplified by polymerase chain reaction to detect amplification products characteristic of the normal gene or of particular gene rearrangements.

The invention is further illustrated by the following non-limiting examples.

#### Example I. Isolation of human MPL receptor cDNAs

Human MPL-P and MPL-K receptor isoform encoding cDNAs were isolated from human erythroid leukemic (HEL) cells (Martin and Papayannopoulou, Science 216: 1233-1235, 1982) by reverse transcriptase polymerase chain reaction (PCR) employing primers made to the published sequence

encoding the amino and carboxyl termini of the receptors (Vigon et al., Proc. Natl. Acad. Sci. USA 89: 5640-5644, 1992). Template HEL cell cDNA was synthesized from poly d(T)-selected poly(A)<sup>+</sup> RNA using primer ZC5499 (SEQ ID NO: 3). Thirteen  $\mu$ l of HEL cell poly(A)<sup>+</sup> RNA at a concentration of 1  $\mu$ g/ $\mu$ l was mixed with 3  $\mu$ l of 20 pmole/ $\mu$ l first strand primer ZC5499 (SEQ ID NO: 3). The mixture was heated at 65° C for 4 minutes and cooled by chilling on ice.

First strand cDNA synthesis was initiated by the addition of 8  $\mu$ l of first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>) (5x SUPERScript™ buffer; GIBCO BRL, Gaithersburg, MD), 4  $\mu$ l of 100 mM dithiothreitol and 3  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The reaction mixture was incubated at 45°C for 4 minutes followed by the addition of 10  $\mu$ l of 200 U/ $\mu$ l of RNase H<sup>-</sup> reverse transcriptase (SUPERScript™ reverse transcriptase; GIBCO BRL) to the RNA-primer mixture. The reaction was incubated at 45° C for 1 hour followed by an incubation at 50° C for 15 minutes. Sixty  $\mu$ l of TE (10 mM Tris:HCl, pH 8.0, 1 mM EDTA) was added to the reaction followed by chromatography through a 400 pore size gel filtration column (CHROMA SPIN+TE-400™; Clontech Laboratories Inc., Palo Alto, CA) to remove excess primer.

First strand HEL cell cDNA was used as a template for the amplification of human MPL-P receptor cDNA using primers corresponding to the region encoding the amino and carboxyl termini of the receptor protein (Vigon et al., *ibid.*). The primers also each incorporated a different restriction enzyme cleavage site to aid in the directional cloning of the amplified product (ZC5746, SEQ ID NO: 4, containing an Eco RI site; ZC5762, SEQ ID NO: 5, containing an Xho I site). A 100  $\mu$ l reaction was set up containing 10 ng of template cDNA, 50 pmoles of each

primer; 200  $\mu$ M of each deoxynucleotide triphosphate (Pharmacia LKB Biotechnology Inc.); 1  $\mu$ l of 10x PCR buffer (Promega Corp., Madison, WI); and 10 units of Taq polymerase (Roche Molecular Systems, Inc., Branchburg, NJ). The polymerase chain reaction was run for 35 cycles (1 minute at 95° C, 1 minute at 60° C and 2 minutes at 72° C with 1 extra second added to each successive cycle) followed by a 10 minute incubation at 72° C.

Human *MPL-K* receptor cDNA was isolated by polymerase chain reaction amplification from HEL cell cDNA in a manner identical to the *MPL-P* receptor cDNA described above, except primer ZC5762 (SEQ ID NO: 5) was replaced with ZC5742 (SEQ ID NO: 6). PCR primer ZC5742 is specific to the 3' terminus of human *MPL-K* cDNA and incorporated an *Xho* I restriction site to facilitate cloning.

The reaction products were extracted twice with phenol/chloroform (1:1), then once with chloroform and were ethanol precipitated. Following digestion with *Eco* RI and *Xho* I, the products were fractionated on a 0.8% low melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp., Rockland, ME). A 1.9 Kb amplified product corresponding to human *MPL-P* receptor cDNA and a 1.7 Kb product corresponding to human *MPL-K* receptor cDNA were recovered from the excised gel slices by digestion of the gel matrix with  $\beta$ -agarase I (New England Biolabs, Inc., Beverly, MA) followed by ethanol precipitation. The cDNAs were subcloned into the vector pBluescript® SK+ (Stratagene Cloning Systems, La Jolla, CA) for validation by sequencing.

#### Example II. Isolation of Mouse *MPL* Receptor cDNA

Spleens from C57BL/KsJ-db/db mice were removed and immediately placed in liquid nitrogen. Total RNA was prepared from spleen tissue using guanidine isothiocyanate (Chirgwin et al., *Biochemistry* 18: 52-94, 1979) followed

by a CsCl centrifugation step. Spleen poly(A)<sup>+</sup> RNA was isolated using oligo d(T) cellulose chromatography (Aviv and Leder, Proc. Natl. Acad. Sci. U.S.A. 69: 1408-1412, 1972).

5           Seven and a half  $\mu$ l of poly d(T)-selected poly(A)<sup>+</sup> mouse spleen RNA at a concentration of 1.7  $\mu$ g/ $\mu$ l was mixed with 3  $\mu$ l of 20 pmole/ $\mu$ l first strand primer ZC6091 (SEQ ID NO: 7) containing a Not I restriction site. The mixture was heated at 65° C for 4 minutes and cooled  
10 by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8  $\mu$ l of 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub> (5x SUPERScript™ buffer; GIBCO BRL), 4  $\mu$ l of 100 mM dithiothreitol and 3  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM  
15 each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc.) to the RNA-primer mixture. The reaction mixture was incubated at 45° C for 4 minutes followed by the addition of 10  $\mu$ l of 200 U/ $\mu$ l RNase H<sup>-</sup> reverse transcriptase (GIBCO BRL). The efficiency of the  
20 first strand synthesis was analyzed in a parallel reaction by the addition of 10  $\mu$ Ci of <sup>32</sup>P- $\alpha$ dCTP to a 10  $\mu$ l aliquot of the reaction mixture to label the reaction for analysis. The reactions were incubated at 45° C for 1 hour followed by an incubation at 50° C for 15 minutes.  
25 Unincorporated <sup>32</sup>P- $\alpha$ dCTP in the labeled reaction was removed by chromatography on a 400 pore size gel filtration column (CHROMA SPIN + TE-400™; Clontech Laboratories Inc.). Unincorporated nucleotides in the unlabeled first strand reaction were removed by twice  
30 precipitating the cDNA in the presence of 8  $\mu$ g of glycogen carrier, 2.5 M ammonium acetate and 2.5 volume ethanol. The unlabeled cDNA was resuspended in 50  $\mu$ l water for use in second strand synthesis. The length of the labeled first strand cDNA was determined by agarose gel  
35 electrophoresis.

Second strand synthesis was performed on first strand cDNA under conditions that promoted first strand priming of second strand synthesis resulting in DNA hairpin formation. The reaction mixture was assembled at room temperature and consisted of 50  $\mu$ l of the unlabeled first strand cDNA, 16.5  $\mu$ l water, 20  $\mu$ l of 5x polymerase I buffer (100 mM Tris: HCl, pH 7.4, 500 mM KCl, 25 mM MgCl<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 1  $\mu$ l of 100 mM dithiothreitol, 2  $\mu$ l of a solution containing 10 mM of each deoxynucleotide triphosphate, 3  $\mu$ l of 5 mM  $\beta$ -NAD, 15  $\mu$ l of 3 U/ $\mu$ l *E. coli* DNA ligase (New England Biolabs Inc., Beverly, MA) and 5  $\mu$ l of 10 U/ $\mu$ l *E. coli* DNA polymerase I (Amersham Corp., Arlington Heights, IL). The reaction was incubated at room temperature for 5 minutes followed by the addition of 1.5  $\mu$ l of 2 U/ $\mu$ l RNase H (GIBCO BRL). A parallel reaction in which a 10  $\mu$ l aliquot of the second strand synthesis mixture was labeled by the addition of 10  $\mu$ Ci <sup>32</sup>P- $\alpha$ dCTP was used to monitor the efficiency of second strand synthesis. The reactions were incubated at 15° C for two hours followed by a 15 minute incubation at room temperature. Unincorporated <sup>32</sup>P- $\alpha$ dCTP in the labeled reaction was removed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories, Inc.) before analysis by agarose gel electrophoresis. The unlabeled reaction was terminated by two extractions with phenol/chloroform and a chloroform extraction followed by ethanol precipitation in the presence of 2.5 M ammonium acetate.

The single-stranded DNA of the hairpin structure was cleaved using mung bean nuclease. The reaction mixture contained 100  $\mu$ l of second strand cDNA, 20  $\mu$ l of 10x mung bean nuclease buffer (Stratagene Cloning Systems, La Jolla, CA), 16  $\mu$ l of 100 mM dithiothreitol, 51.5  $\mu$ l of water and 12.5  $\mu$ l of a 1:10 dilution of mung bean nuclease (Promega Corp.; final concentration 10.5 U/ $\mu$ l) in mung bean nuclease dilution buffer. The reaction was incubated at



37° C for 15 minutes. The reaction was terminated by the addition of 20  $\mu$ l of 1 M Tris: HCl, pH 8.0 followed by sequential phenol/chloroform and chloroform extractions as described above. Following the extractions, the DNA was  
5 precipitated in ethanol and resuspended in water.

The resuspended cDNA was blunt-ended with T4 DNA polymerase. The cDNA, which was resuspended in 190  $\mu$ l of water, was mixed with 50  $\mu$ l 5x T4 DNA polymerase buffer (250 mM Tris:HCl, pH 8.0, 250 mM KCl, 25 mM  $MgCl_2$ ), 3  $\mu$ l  
10 0.1 M dithiothreitol, 3  $\mu$ l of a solution containing 10 mM of each deoxynucleotide triphosphate and 4  $\mu$ l of 1 U/ $\mu$ l T4 DNA polymerase (Boehringer Mannheim Corp., Indianapolis, IN). After an incubation of 1 hour at 10° C, the reaction was terminated by the addition of 10  $\mu$ l of 0.5 M EDTA  
15 followed by serial phenol/chloroform and chloroform extractions as described above. The DNA was chromatographed through a 400 pore size gel filtration column (Clontech Laboratories Inc., Palo Alto, CA) to remove trace levels of protein and to remove short cDNAs  
20 less than -400 bp in length. The DNA was ethanol precipitated in the presence of 12  $\mu$ g glycogen carrier and 2.5 M ammonium acetate and was resuspended in 10  $\mu$ l of water. Based on the incorporation of  $^{32}P$ - $\alpha$ dCTP, the yield of cDNA was estimated to be -2  $\mu$ g from a starting mRNA  
25 template of 12.5  $\mu$ g.

*Eco* RI adapters were ligated onto the 5' ends of the cDNA to enable cloning into a lambda phage vector. A 10  $\mu$ l aliquot of cDNA (-2 $\mu$ g) and 10  $\mu$ l of 65 pmole/ $\mu$ l of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc.) were  
30 mixed with 2.5  $\mu$ l 10x ligase buffer (Promega Corp.), 1  $\mu$ l of 10 mM ATP and 2  $\mu$ l of 15 U/ $\mu$ l T4 DNA ligase (Promega Corp.). The reaction was incubated overnight (-18 hours) at a temperature gradient of 0° C to 18° C. The reaction was further incubated overnight at 12° C. The reaction  
35 was terminated by the addition of 75  $\mu$ l of water and 10  $\mu$ l of 3 M Na acetate, followed by incubation at 65° C for 30

minutes. After incubation, the cDNA was extracted with phenol/chloroform and chloroform as described above and precipitated in the presence of 2.5 M ammonium acetate and 1.2 volume of isopropanol. Following centrifugation, the  
5 cDNA pellet was washed with 70% ethanol, air dried and resuspended in 89  $\mu$ l water.

To facilitate the directional cloning of the cDNA into a lambda phage vector, the cDNA was digested with Not I, resulting in a cDNA having 5' Eco RI and 3'  
10 Not I cohesive ends. The Not I restriction site at the 3' end of the cDNA had been previously introduced through primer ZG6091 (SEQ ID NO: 7). Restriction enzyme digestion was carried out in a reaction containing 89  $\mu$ l of cDNA described above, 10  $\mu$ l of 6 mM Tris:HCl, 6 mM MgCl<sub>2</sub>,  
15 150 mM NaCl, 1 mM DTT (10x D buffer; Promega Corp., Madison, WI) and 1  $\mu$ l of 12 U/ $\mu$ l Not I (Promega Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by serial phenol/chloroform and chloroform extractions. The cDNA was ethanol  
20 precipitated, washed with 70% ethanol, air dried and resuspended in 20  $\mu$ l of 1x gel loading buffer (10 mM Tris:HCl, pH 8.0, 1 mM EDTA, 5% glycerol and 0.125% bromophenol blue).

The resuspended cDNA was heated to 65°C for 5  
25 minutes, cooled on ice and electrophoresed on a 0.8% low melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp.). Unincorporated adapters and cDNA below 1.6 Kb in length were excised from the gel. The electrodes were reversed, and the cDNA was electrophoresed until  
30 concentrated near the lane origin. The area of the gel containing the concentrated cDNA was excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water (300  $\mu$ l) approximately three times the volume of the gel slice was  
35 added to the tube, and the agarose was melted by heating to 65° C for 15 minutes. Following equilibration of the

sample to 42° C, 10  $\mu$ l of 1 U/ $\mu$ l  $\beta$ -agarase I (New England Biolabs, Inc.) was added, and the mixture was incubated for 90 minutes to digest the agarose. After incubation, 40  $\mu$ l of 3 M Na acetate was added to the sample, and the  
5 mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose. The cDNA in the supernatant was ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 37  $\mu$ l of water for  
10 the kinase reaction to phosphorylate the ligated Eco RI adapters.

To the 37  $\mu$ l cDNA solution described above was added 10  $\mu$ l 10x ligase buffer (Stratagene Cloning Systems), and the mixture was heated to 65° C for 5 minutes. The  
15 mixture was cooled on ice, and 5  $\mu$ l 10 mM ATP and 3  $\mu$ l of 10 U/ $\mu$ l T4 polynucleotide kinase (Stratagene Cloning Systems) were added. The reaction was incubated at 37°C for 45 minutes and was terminated by heating to 65° C for 10 minutes followed by serial extractions with  
20 phenol/chloroform and chloroform. The phosphorylated cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate, washed with 70% ethanol, air dried and resuspended in 12.5  $\mu$ l water. The concentration of the phosphorylated cDNA was estimated to be ~40 fmole/ $\mu$ l.

The resulting cDNA was cloned into the lambda phage vector  $\lambda$ ExCell™ (Pharmacia LKB Biotechnology Inc.), purchased predigested with Eco RI and Not I and dephosphorylated. Ligation of cDNA to vector was carried out in a reaction containing 2  $\mu$ l of 20 fmole/ $\mu$ l prepared  
25  $\lambda$ ExCell™ phage arms, 4  $\mu$ l of water, 1  $\mu$ l 10x ligase buffer (Promega Corp.), 2  $\mu$ l of 40 fmole/ $\mu$ l cDNA and 1  $\mu$ l of 15 U/ $\mu$ l T4 DNA ligase (Promega Corp.). Ligation was carried out at 4° C for 48 hours. Approximately 50% of the ligation mixture was packaged into phage using GIGAPACK®  
30 II Gold packaging extract (Stratagene Cloning Systems) according to the directions of the vendor. The resulting  
35

cDNA library contained over  $1.5 \times 10^7$  independent recombinants with background levels of insertless phage of less than 1.5%.

5 A  $^{32}\text{P}$ -labeled human *MPL-K* receptor cDNA probe was used to isolate mouse *MPL* receptor cDNA from the mouse spleen cDNA phage library. The cDNA library was plated on SURE<sup>®</sup> strain of *E. coli* cells (Stratagene Cloning Systems) at a density of 40,000 to 50,000 PFU/150 mm diameter plate. Phage plaques from thirty-three plates were  
10 transferred onto nylon membranes (Hybond N<sup>™</sup>; Amersham Corp., Arlington Heights, IL) and processed according to the directions of the manufacturer. The processed filters were baked for 2 hours at 80° C in a vacuum oven followed by several washes at 70° C in wash buffer (0.25 x SSC, 0.25% SDS, 1 mM EDTA) and prehybridized overnight at 65° C  
15 in hybridization solution (5x SSC, 5x Denhardt's solution, 0.1% SDS, 1 mM EDTA and 100 µg/ml heat denatured salmon sperm DNA) in a hybridization oven (model HB-2; Techne Inc., Princeton, NJ). Following prehybridization, the hybridization solution was discarded and replaced with  
20 fresh hybridization solution containing approximately  $2 \times 10^6$  cpm/ml of  $^{32}\text{P}$ -labeled human *MPL-K* cDNA prepared by the use of a commercially available labeling kit (MEGAPRIME<sup>™</sup> kit; Amersham Corp., Arlington Heights, IL). The probe  
25 was denatured at 98° C for 5 minutes before being added to the hybridization solution. Hybridization was at 65° C overnight. The filters were washed at 55° C in wash buffer (0.25 x SSC, 0.25% SDS, 1 mM EDTA) and were autoradiographed with intensifying screens for 4 days at -  
30 70° C on XAR-5 film (Kodak Inc., Rochester, NY). Employing the autoradiograph as template, agar plugs were recovered from regions of the plates corresponding to primary signals and were soaked in SM (0.1 M NaCl; 50 mM Tris:HCl, pH 7.5, 0.02% gelatin) to elute phage for plaque  
35 purification. Seven plaque-purified phages were isolated that carried inserts hybridizing to the human *MPL-K*

receptor probe. The phagemids contained within the  $\lambda$  ExCell™ phage were recovered using the *in vivo* recombination system in accordance with the directions of the vendor. The identity of the cDNA inserts was confirmed by DNA sequencing.

The isolated clones encoded a protein exhibiting a high degree of sequence identity to human MPL-P receptor and to a recently reported mouse MPL receptor (Skoda et al., EMBO J. 12: 2645-2653, 1993). The seven clones fell into two classes differing from each other by three clones having a deletion of sequences encoding a stretch of 60 amino acid residues near the N-terminus. The cDNA encoding the protein without the deletion was referred to as mouse Type I MPL receptor cDNA. Type II receptor cDNA lacked sequences encoding Type I receptor residues 131 to 190 of SEQ ID NO: 17. In addition, Type I and II receptors differed from the reported mouse MPL receptor sequence (Skoda et al., *ibid.*) by the presence of a sequence encoding the amino acid residues Val-Arg-Thr-Ser-Pro-Ala-Gly-Glu (SEQ ID NO: 9) inserted after amino acid residue 222 and by a substitution of a glycine residue for serine at position 241 (positions refer to the Type I mouse receptor).

Type I and II mouse MPL receptor cDNAs were subcloned into the plasmid vector pHZ-1 for expression in mammalian cells. Plasmid pHZ-1 is an expression vector that may be used to express protein in mammalian cells or in a frog oocyte translation system from mRNAs that have been transcribed *in vitro*. The pHZ-1 expression unit comprises the mouse metallothionein-1 promoter, the bacteriophage T7 promoter flanked by multiple cloning banks containing unique restriction sites for insertion of coding sequences, the human growth hormone terminator and the bacteriophage T7 terminator. In addition, pHZ-1 contains an *E. coli* origin of replication; a bacterial beta lactamase gene; a mammalian selectable marker

expression unit comprising the SV40 promoter and origin, a neomycin resistance gene and the SV40 transcription terminator. To facilitate directional cloning into pHZ-1, a polymerase chain reaction employing appropriate primers was used to create an *Eco* RI site and a *Xho* I site upstream from the translation initiation codon and downstream from the translation termination codon, respectively. The polymerase chain reaction was carried out in a mixture containing 10  $\mu$ l 10x ULTMA™ DNA polymerase buffer (Roche Molecular Systems, Inc., Branchburg, NJ), 6  $\mu$ l of 25 mM  $MgCl_2$ , 0.2  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia LKB Biotechnology Inc.), 2.5  $\mu$ l of 20 pmole/ $\mu$ l primer ZC6603 (SEQ ID NO: 8), 2.5  $\mu$ l of 20 pmole/ $\mu$ l primer ZC5762 (SEQ ID NO: 5), 32.8  $\mu$ l of water, 1  $\mu$ l of an early log phase bacterial culture harboring either a Type I or a Type II mouse MPL receptor plasmid and 1  $\mu$ l of 6 U/ $\mu$ l DNA polymerase (ULTMA™ polymerase; Roche Molecular Systems, Inc., Branchburg, NJ). AmpliWax™ (Roche Molecular Systems, Inc.) was employed in the reaction according to the directions of the vendor. The polymerase chain reaction was run for 25 cycles (1 minute at 95° C, 1 minute at 55° C and 3 minutes at 72° C) followed by a 10 minute incubation at 72° C. The amplified products were serially extracted with phenol/chloroform and chloroform, then ethanol precipitated in the presence of 6  $\mu$ g glycogen carrier and 2.5 M ammonium acetate. The pellets were resuspended in 87  $\mu$ l of water to which was added 10  $\mu$ l of 10 x H buffer (Boehringer Mannheim Corp.), 2  $\mu$ l of 10 U/ $\mu$ l *Eco* RI (Boehringer Mannheim) and 1  $\mu$ l of 40 U/ $\mu$ l *Xho* I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by heating to 65° C for 15 minutes and chromatographed through a 400 pore size gel filtration column (CHROMA SPIN + TE-400™; Clontech Laboratories Inc.).

The isolated receptor inserts described above were ligated into *Eco* RI and *Xho* I digested and dephosphorylated pHZ-1 vector. The ligation reaction contained 1  $\mu$ l of 50 ng/ $\mu$ l prepared pHZ-1 vector, 5  $\mu$ l of 5 ng/ $\mu$ l cDNA insert, 2  $\mu$ l of 10x ligase buffer (Promega Corp.), 11.75  $\mu$ l water and 0.25  $\mu$ l of 4 U/ $\mu$ l T4 DNA ligase (Stratagene Cloning Systems). Ligation was carried out at 10° C overnight. The ligated DNAs were transfected into *E. coli* (MAX EFFICIENCY DH10B™ competent cells; GIBCO BRL) in accordance with the vendor's directions. The validity of Type I and Type II mouse *MPL* and human *MPL-P* receptor inserts in pHZ-1 was confirmed by DNA sequencing. The resulting plasmids pSLmpl-8 and pSLmpl-9 carried the mouse Type II and Type I *MPL* receptor cDNAs, respectively. Plasmid pSLmpl-44 carried the human *MPL-P* cDNA insert.

Example III. Construction of BaF3 Cell Lines Expressing MPL Receptors

BaF3, an interleukin-3 dependent pre-lymphoid cell line derived from murine bone marrow (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol. 6: 4133-4135, 1986), was maintained in complete media (RPMI 1640 medium (JRH Bioscience Inc., Lenexa, KS) supplemented with 10% heat-inactivated fetal calf serum, 4% conditioned media from cultured WEHI-3 cells (Becton Dickinson Labware, Bedford, MA), 2mM L-glutamine, 2-mercaptoethanol (1:280,000 final conc.) and PSN antibiotics (GIBCO BRL)). Cesium chloride purified plasmids pSLmpl-8, pSLmpl-9 and pSLmpl-44 were linearized at the *Nde* I site prior to electroporation into BaF3 cells. BaF3 cells for electroporation were washed once in RPMI 1640 media and resuspended in RPMI 1640 media at a cell density of 10<sup>7</sup> cells/ml. One ml of resuspended BaF3 cells was mixed with 30  $\mu$ g of each of the linearized plasmid DNAs and transferred to separate disposable electroporation chambers (GIBCO BRL). Following a 15

minute incubation at room temperature the cells were given two serial shocks (800  $\mu$ Fad/300 V.; 1180  $\mu$ Fad/300 V.) delivered by an electroporation apparatus (CELL-PORATOR™; GIBCO BRL). After a 5 minute recovery time, the  
5 electroporated cells were transferred to 10 ml of complete media and placed in an incubator for 15-24 hours (37° C, 5% CO<sub>2</sub>). The cells were then spun down and resuspended in 10 ml of complete media containing 1600  $\mu$ g/ml G418 and plated at limiting dilutions in 96-well tissue culture  
10 plates to isolate G418-resistant clones. Expression of MPL receptors in G418-resistant BaF3 clones was inferred by Northern blot analysis of BaF3 mRNA for the presence of MPL receptor transcript. A cell line designated BaF3/MPLR1.1 was found to express high levels of Type I  
15 mouse MPL receptor mRNA and was used for subsequent assay for MPL ligand activity in conditioned media of transfected BHK 570 cells. A BaF3 cell line expressing Type II receptor mRNA was designated as BaF3/MPLR2.

20 Example IV. Production of Soluble Mouse MPL Receptor

A mammalian expression plasmid encoding soluble mouse Type I MPL receptor (pLDmpl-53) was produced by combining DNA segments from pSLmpl-9, a mammalian  
expression plasmid containing the cDNA encoding full-  
25 length mouse Type I MPL receptor described above, with a DNA segment from pSLmpl-26, an expression plasmid constructed to produce the soluble mouse Type I MPL receptor in bacteria.

A cDNA segment encoding mouse Type I MPL soluble  
30 receptor was isolated by PCR employing primers ZC6704 (SEQ ID NO: 10) and ZC6703 (SEQ ID NO: 11) using full-length receptor plasmid pSLmpl-9 as template. To facilitate directional cloning, primers ZC6704 and ZC6703 incorporated Eco RI and Xho I restriction sites at their  
35 respective 5' ends. Primer ZC6703 also encoded an inframe consensus target sequence for protein kinase to enable in



vitro labeling of the purified soluble receptor with  $^{32}\text{P}$   $\gamma$ -ATP (Li et al., Proc. Natl. Acad. Sci. U.S.A. 86: 558-562, 1989). The PCR was carried out in a mixture containing 10  $\mu\text{l}$  10x ULTMA<sup>TM</sup> DNA polymerase buffer (Roche Molecular Systems, Inc.), 6  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , 0.2  $\mu\text{l}$  of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia LKB Biotechnology Inc.), 11  $\mu\text{l}$  of 4.55 pmole/ $\mu\text{l}$  primer ZC6704 (SEQ ID NO: 10), 21  $\mu\text{l}$  of 2.43 pmole/ $\mu\text{l}$  primer ZC6703 (SEQ ID NO: 11), 50.3  $\mu\text{l}$  of water, 1  $\mu\text{l}$  50 ng/ $\mu\text{l}$  *Hind* III and *Xba* I digested pSLmpl-9 and 1  $\mu\text{l}$  of 6 U/ $\mu\text{l}$  ULTMA<sup>TM</sup> DNA polymerase (Roche Molecular Systems, Inc.). AmpliWax<sup>TM</sup> (Roche Molecular Systems, Inc.) was employed in the reaction according to the directions of the vendor. The polymerase chain reaction was run for 3 cycles (1 minute at 95° C, 1 minute at 50° C and 2 minutes at 72° C) followed by 11 cycles at increased hybridization stringency (1 minute at 95° C, 30 seconds at 55° C and 2 minutes at 72° C) followed by a 10 minute incubation at 72° C. The amplified product was serially extracted with phenol/chloroform and chloroform followed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories, Inc.). The PCR product was ethanol precipitated in the presence of 20  $\mu\text{g}$  glycogen carrier and 2.5 M ammonium acetate. The pellet was resuspended in 32  $\mu\text{l}$  of water. To 16  $\mu\text{l}$  of the resuspended PCR product was added 2  $\mu\text{l}$  10x H buffer (Boehringer Mannheim Corp.), 1  $\mu\text{l}$  of 10 U/ $\mu\text{l}$  *Eco* RI (Boehringer Mannheim Corp.) and 1  $\mu\text{l}$  of 40 U/ $\mu\text{l}$  *Xho* I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. Digestion was terminated by heating to 65° C for 15 minutes and was purified on a 0.7% low-melt agarose gel. Fragment recovery from low-melt agarose was done by digestion of the gel matrix with  $\beta$ -agarase I (New England Biolabs).

The resulting PCR product encoded the N-terminal extracellular domain of mouse Type I MPL receptor (residues 27 to 480 of SEQ ID NO: 17). In the absence of the putative receptor trans-membrane domain (residues 483 to 504 of SEQ ID NO: 17) the expressed protein is expected to be secreted in the presence of a suitable signal peptide. A mouse Type II soluble MPL receptor encoding cDNA was obtained using the PCR conditions described above except that pSLmpl-8 was used as template. The validity of both receptor fragments was confirmed by DNA sequencing.

The soluble mouse Type I and Type II MPL receptor encoding DNA fragments were cloned into *Eco* RI and *Xho* I digested vector pOmpA2-5 to yield pSLmpl-26 and pSLmpl-27, respectively. Plasmid pOmpA2-5 is a modification of pOmpA2 (Ghrayab et al., EMBO J. 3: 2437-2442, 1984), a bacterial expression vector designed to target the recombinant protein to the periplasmic space. pOmpA2-5 was constructed by replacement of a 13 bp sequence between the *Eco* RI and *Bam* HI sites of pOmpA2 with a synthetic 42 bp sequence. The sequence was created by annealing of two 42 nucleotide complementary oligonucleotides (ZC6707, SEQ ID NO: 12; ZC 6706, SEQ ID NO: 13), which when base paired formed *Eco* RI and *Bam* HI cohesive ends, facilitating directional cloning into *Eco* RI and *Bam* HI digested pOmpA2. Within the inserted sequence is an *Xho* I site inframe with respect to a bacterial leader sequence and to the mouse MPL soluble receptor encoding cDNAs described above, as well as an inframe tract of 6 histidine codons located 3' of the *Xho* I site to enable the recombinant protein to be purified by metal chelation affinity chromatography (Houchuli et al., Bio/Technol. 6: 1321-1325, 1988). Following the sequence encoding the histidine tract was an inframe termination codon. The validity of the pOmpA2-5, pSLmpl-26 and pSLmpl-27 was confirmed by DNA sequencing.

pLDmpl-53, a mammalian expression plasmid producing soluble mouse Type I MPL receptor, was constructed by combining DNA segments from pSLmpl-9 and pSLmpl-26 into expression vector pHZ-200 (pHZ-1 in which a dihydrofolate reductase sequence was substituted for the neomycin resistance gene). The 1164 bp *Eco* RI/*Bam* HI cDNA fragment from pSLmpl-9 replaced the mammalian signal sequence deleted during the construction of bacterial expression plasmid pSLmpl-26. The 416 bp *Bam* HI fragment from pSLmpl-26 supplied the coding sequence for the carboxy-terminal portion of the soluble MPL receptor, the kinase labeling domain, the poly-histidine tract and the translation terminator. The two fragments were gel purified and cloned into the *Eco* RI/*Bam* HI sites of pBluescript® KS+ (Stratagene Cloning Systems) to yield plasmid pBS8.76LD-5. Correct orientation of the the 416 bp pSLmpl-26 derived *Bam* HI fragment with respect to the 1164 bp pSLmpl-9 derived *Eco* RI/*Bam* HI fragment in pBS8.76LD-5 was determined by PCR using primers ZC 6603 (SEQ ID NO: 8) and ZC 6703 (SEQ ID NO: 11). The *Xba* I site within the poly-linker sequence of pBS8.76LD-5 enabled the reconstituted receptor cDNA to be excised as a 1.5 kb *Eco* RI/*Xba* I fragment for cloning into pHZ-200 following digestion of the vector with *Eco* RI and *Xba* I. The resulting mammalian expression plasmid, pLDmpl-53, was prepared in large scale for transfection into BHK cells.

Twenty micrograms of purified pLDmpl-53 plasmid was transfected into BHK 570 cells using the calcium phosphate precipitation method. After 5 hours, the cells were shocked with 15% glycerol for 3 minutes to facilitate uptake of DNA. Fresh growth media was added overnight. The following day the cells were split at various dilutions, and selection media containing 1  $\mu$ M methotrexate was added. After approximately two weeks, discrete, methotrexate-resistant colonies were visible. Resistant colonies were either pooled or maintained as distinct

clones. Spent media from the pooled colonies was immediately tested for presence of soluble MPL receptor protein.

Soluble MPL receptor protein was isolated through the interaction of the poly-histidine tract present on the carboxy-terminal of the protein with a metal chelation resin containing immobilized  $\text{Ni}^{2+}$  (HIS-BIND™; Novagen, Madison, WI). Serum-free spent culture media from the pLDmpl-53 pool was passed over the resin, and bound protein was eluted with 1 M imidazole. SDS-PAGE analysis revealed a single band at ~67 kDa. This protein was subjected to N-terminal amino acid analysis and confirmed to be mouse MPL receptor.

Soluble mouse MPL receptor was purified from a pool of BHK transfectants, which had been transfected with the soluble mouse Type I MPL receptor expressing plasmid pLDmpl-53. The purified soluble receptor was immobilized on CNBr-activated SEPHAROSE™ 4B (Pharmacia LKB Biotechnology, Inc.) matrix essentially as directed by the manufacturer and used for affinity purification of the MPL activity in conditioned media of 24-11-5 cells. The affinity matrix was packed in a XK16 column (Pharmacia LKB Biotechnology Inc.). Conditioned media from 24-11-5 cells were concentrated on a 10 Kd cut off hollow fiber membrane (A/G Technology Corp., Needham, MA) and loaded onto the bottom of the MPL receptor affinity column at a flow rate of 1 ml/minute. The column was washed with phosphate buffered saline (PBS) containing 0.5 M NaCl and 0.01% sodium azide. MPL activity was eluted from the column with 3M potassium thiocyanate (Sigma Chemical Company, St. Louis, MO) at a flow rate of 0.5 ml/minute. Potassium thiocyanate was removed by dialysis against PBS. Active fractions were identified by MTT proliferation assay (disclosed in Example VII).

Example V. Isolation and Characterization of a MPL Receptor Ligand Expressing Cell Line

BaF3/MPLR1.1 cells are IL-3 dependent cells expressing a stably transfected Type I mouse MPL receptor.

5 A mutagenesis and selection scheme was devised to isolate cell lines expressing the MPL receptor ligand by mutagenizing BaF3/MPLR1.1 cells, and selecting for autocrine growth in the absence of exogenous IL-3.

Approximately  $1.2 \times 10^6$  BaF3/MPLR1.1 cells were  
10 pelleted and washed with GM (RPMI 1640 media supplemented with 2-mercaptoethanol (1:240,000 final concentration), 2 mM L-glutamine, 110  $\mu$ g/ml sodium pyruvate, 50  $\mu$ g/ml G418 and 10% heat inactivated fetal bovine serum). The cells were resuspended in 2 ml of GM containing 0.15% (v/v) of  
15 the mutagen 2-ethylmethanesulfonate (EMS) and incubated for 2 hours at 37°C. After incubation, the cells were washed once in PBS and once in GM and plated onto 10 cm plates at density of approximately 40,000 cells/ml in GM supplemented with 5% WEHI-3 conditioned media (Becton  
20 Dickinson Labware, Bedford, MA) as a source of IL-3. The cells were allowed a recovery period of seven days incubated at 37°C under 5% CO<sub>2</sub> before selection for IL-3 independent growth. Following the recovery period, the culture was dense with viable cells. The cells were  
25 washed with GM and were cultured in GM in the absence of WEHI-3 conditioned media. After eleven days of selection, small numbers of viable cells were observed. The viable cell density of the IL-3 independent culture was estimated to be 250 cells/ml. One ml of the IL-3  
30 independent culture was plated onto each of 19 wells of a 24-well culture plate for further characterization.

Conditioned media from the above IL-3 growth independent BaF3/MPLR1.1 cells were assayed for  
proliferative activity on BaF3/MPLR cells. Conditioned  
35 media from all nineteen IL-3 growth independent pools were found to have activity in the MTT proliferation assay

(disclosed in Example VII). The positive media were reassayed for proliferative activity in the presence of 2  $\mu$ g/ml rat anti-mouse IL-3, anti-mouse IL-4 or in the presence of both neutralizing antibodies (Pharmingen, San Diego, CA) to identify IL-3 growth independent mutants expressing those cytokines. (In a previous experiment, it was found that BaF3 cells also responded to IL-4.) Only conditioned medium from cells from plate #11 (designated "24-11" cells) was found to have activity that was not neutralized by IL-3 or IL-4 antibodies.

The mutagenesis and selection scheme described above was applied to five other BaF3/MPLR1 clones (BaF3/MPLR1 clones # 4, 9, 12, 15 and 18, designated as BaF3/MPLR1.4, .9, .12, .15 and .18, respectively). Seventeen isolates were found to have conditioned media which stimulated proliferation of BaF3/MPLR1 cells. Activity of all the media was found to be neutralized by anti-IL-3 or IL-4 antibodies alone or in combination. These clones were not characterized further.

The proliferative activity of conditioned media from the 24-11 pool was characterized in detail. The 24-11 pool was subdivided into nineteen subpools, and conditioned media were retested for activity. All nineteen subpools (i.e. 24-11-1 thru 24-11-19) stimulated proliferation of IL-3 growth dependent BaF3/MPLR1 cells in the absence of exogenous IL-3. The activity was not inhibited by IL-3 or IL-4 neutralizing antibodies or by a combination of both antibodies.

Two experiments were performed to determine the specificity of the 24-11 activity. The conditioned media were assayed for proliferative activity on control BaF3 cells that do not express the MPL receptor. In the absence of exogenous IL-3, proliferation of control BaF3 cells was not observed in the conditioned media from any of the nineteen 24-11 subpools. In a second experiment, proliferative activity was assayed for inhibition by

purified soluble MPL receptor. BaF3/MPLR1 cells were cultured in GM media supplemented with 50% 24-11 conditioned media. To each sample was added Type I mouse soluble MPL receptor to a final concentration of 0.0, 0.625, 1.25, 2.5 or 5.0  $\mu\text{g/ml}$ . The results were scored 4 days later by MTT cell proliferation assay. The proliferative activity of the 24-11 conditioned media was completely blocked at 0.625 to 1.25  $\mu\text{g/ml}$  soluble MPL receptor. Soluble receptor concentrations that completely inhibited activity had no effect on IL-3 or IL-4 stimulation of BaF3/MPLR1 cells. The results indicated that soluble MPL receptor competed for the stimulatory activity of 24-11 media and were consistent with the hypothesis that 24-11 cells expressed the MPL receptor ligand.

Clones derived from 24-11 cells were isolated by plating at limiting dilutions. One clone, designated 24-11-5 #3, showed a high level of proliferative activity in its conditioned media relative to the 24-11 pool. The proliferative activity was found to be equal to a 1:2000 dilution of conditioned media from WEHI-3 cells (Becton Dickinson Labware).

#### Example VI. Construction of 24-11-5#3 cDNA library

Total RNA was prepared from  $2.7 \times 10^8$  24-11-5 #3 cells using guanidine isothiocyanate followed by CsCl centrifugation (Chirgwin et al., *ibid.*). Poly(A)<sup>+</sup> RNA was isolated using an OLIGOTEX-dT-mRNA isolation kit (Qiagen Inc., Chatsworth, CA) following the manufacturer's instructions.

First strand cDNA from 24-11-5#3 cells was synthesized in 4 separate parallel reactions. Each reaction contained 7  $\mu\text{l}$  of poly d(T)-selected poly(A)<sup>+</sup> 24-11-5#3 RNA at a concentration of 1.6  $\mu\text{g}/\mu\text{l}$  and 2.5  $\mu\text{l}$  of 20 pmole/ $\mu\text{l}$  first strand primer ZC6172 (SEQ ID NO: 14) containing an Xho I restriction site. The mixture was

heated at 65°C for 4 minutes and cooled by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8 µl of first strand buffer (5x SUPERScript™ buffer; GIBCO BRL), 4 µl of 100 mM dithiothreitol and 2 µl of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc.) to the RNA-primer mixture. The reaction mixture was incubated at 45° C for 4 minutes followed by the addition of 10 µl of 200 U/µl RNase H<sup>-</sup> reverse transcriptase (GIBCO BRL). The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10 µCi of <sup>32</sup>P-αdCTP to a 10 µl aliquot from one of the reaction mixtures to label the reaction for analysis. The reactions were incubated at 45° C for 1 hour followed by an incubation at 50° C for 15 minutes. Unincorporated <sup>32</sup>P-αdCTP in the labeled reaction was removed by chromatography on a 400 pore size gel filtration column (Clontech Laboratories). The unlabeled first strand reactions were pooled, and unincorporated nucleotides were removed by twice precipitating the cDNA in the presence of 32 µg of glycogen carrier, 2.5 M ammonium acetate and 2.5 volume ethanol. The unlabeled cDNA was resuspended in 144 µl water for use in second strand synthesis. The length of labeled first strand cDNA was determined by agarose gel electrophoresis.

Second strand synthesis was performed on the first strand cDNA under conditions that promoted first strand priming of second strand synthesis resulting in DNA hairpin formation. Three separate parallel second strand reactions were performed. Each second strand reaction contained 48 µl of the unlabeled first strand cDNA, 16.5 µl of water, 20 µl of 5x polymerase I buffer (100 mM Tris: HCl, pH 7.4, 500 mM KCl, 25 mM MgCl<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 1 µl of 100 mM dithiothreitol, 1 µl of a solution containing 10 mM of each deoxynucleotide triphosphate, 3 µl of 5 mM β-NAD, 1 µl of 3 U/µl *E. coli* DNA ligase (New England Biolabs



Inc.) and 5  $\mu$ l of 10 U/ $\mu$ l *E. coli* DNA polymerase I (Amersham Corp.). The reaction was assembled at room temperature and was incubated at room temperature for 5 minutes followed by the addition of 1.5  $\mu$ l of 2 U/ $\mu$ l RNase H (GIBCO BRL). A 10  $\mu$ l aliquot from one of the second strand synthesis reactions was labeled by the addition of 10  $\mu$ Ci  $^{32}$ P- $\alpha$ dCTP to monitor the efficiency of second strand synthesis. The reactions were incubated at 15° C for two hours followed by a 15 minute incubation at room temperature. Unincorporated  $^{32}$ P- $\alpha$ dCTP in the labeled reaction was removed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories) before analysis by agarose gel electrophoresis. The unlabeled reactions were pooled and extracted with phenol/chloroform and chloroform followed by ethanol precipitation in the presence of 2.5 M ammonium acetate.

The single-stranded DNA of the hairpin structure was cleaved using mung bean nuclease. The reaction mixture contained 100  $\mu$ l of second strand cDNA, 20  $\mu$ l of 10x mung bean nuclease buffer (Stratagene Cloning Systems), 16  $\mu$ l of 100 mM dithiothreitol, 48  $\mu$ l of water, 10  $\mu$ l of mung bean nuclease dilution buffer (Stratagene Cloning Systems) and 6  $\mu$ l of 50 U/ $\mu$ l mung bean nuclease (Promega Corp.). The reaction was incubated at 37° C for 30 minutes. The reaction was terminated by the addition of 20  $\mu$ l of 1 M Tris: HCl, pH 8.0 followed by sequential phenol/chloroform and chloroform extractions as described above. Following the extractions, the DNA was precipitated in ethanol and resuspended in water.

The resuspended cDNA was blunt-ended with T4 DNA polymerase. The cDNA, which was resuspended in 188  $\mu$ l of water, was mixed with 50  $\mu$ l 5x T4 DNA polymerase buffer (250 mM Tris:HCl, pH 8.0, 250 mM KCl, 25 mM MgCl<sub>2</sub>), 3  $\mu$ l 0.1 M dithiothreitol, 4  $\mu$ l of a solution containing 10 mM of each deoxynucleotide triphosphate and 5  $\mu$ l of 1 U/ $\mu$ l T4 DNA polymerase (Boehringer Mannheim Corp.). After an

incubation of 30 minutes at 15° C, the reaction was terminated by the addition of 10  $\mu$ l of 0.5 M EDTA followed by serial phenol/chloroform and chloroform extractions as described above. The DNA was chromatographed through a  
5 400 pore size gel filtration column (Clontech Laboratories Inc.) to remove trace levels of protein and to remove short cDNAs less than -400 bp in length. The DNA was ethanol precipitated in the presence of 10  $\mu$ g glycogen carrier and 2.5 M ammonium acetate and was resuspended  
10 15  $\mu$ l of water. Based on the incorporation of  $^{32}$ P- $\alpha$ dCTP, the yield of cDNA was estimated to be -8  $\mu$ g from a starting mRNA template of 40  $\mu$ g.

*Eco* RI adapters were ligated onto the 5' ends of the cDNA described above to enable cloning into an  
15 expression vector. A 10  $\mu$ l aliquot of cDNA (-5  $\mu$ g) and 21  $\mu$ l of 65 pmole/ $\mu$ l of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc.) were mixed with 4  $\mu$ l 10x ligase buffer (Promega Corp.), 3  $\mu$ l of 10 mM ATP and 3  $\mu$ l of 15 U/ $\mu$ l T4 DNA ligase (Promega Corp.). The reaction was incubated  
20 overnight (-48 hours) at 9° C. The reaction was terminated by the addition of 140  $\mu$ l of water, 20  $\mu$ l of 10x H buffer (Boehringer Mannheim Corp.) and incubation at 65° C for 40 minutes. After incubation, the cDNA was extracted with phenol/chloroform and chloroform as described above and  
25 precipitated in the presence of 2.5 M ammonium acetate and 1.2 volume of isopropanol. Following centrifugation, the cDNA pellet was washed with 70% ethanol, air dried and resuspended in 89  $\mu$ l water.

To facilitate the directional cloning of the  
30 cDNA into an expression vector, the cDNA was digested with *Xho* I, resulting in a cDNA having a 5' *Eco* RI cohesive end and a 3' *Xho* I cohesive end. The *Xho* I restriction site at the 3' end of the cDNA had been previously introduced using the ZC6172 primer (SEQ ID NO: 14). Restriction  
35 enzyme digestion was carried out in a reaction mixture containing 89  $\mu$ l of cDNA described above, 10  $\mu$ l of 10x H

buffer (Promega Corp.) and 1.5  $\mu$ l of 40 U/ $\mu$ l Xho I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by serial phenol/chloroform and chloroform extractions and chromatography through a 400 pore size gel filtration column (Clontech Laboratories Inc.).

The cDNA was ethanol precipitated, washed with 70% ethanol, air dried and resuspended in 20  $\mu$ l of 1x gel loading buffer (10 mM Tris:HCl, pH 8.0, 1 mM EDTA, 5% glycerol and 0.125% bromphenol blue). The resuspended cDNA was heated to 65° C for 5 minutes, cooled on ice and electrophoresed on a 0.8% low melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp.). The contaminating adapters and cDNA below 0.5 Kb in length were excised from the gel. The electrodes were reversed, and the cDNA was electrophoresed until concentrated near the lane origin. The area of the gel containing the concentrated cDNA was excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water approximately three times the volume of the gel slice (300  $\mu$ l) was added to the tube, and the agarose was melted by heating to 65° C for 15 minutes. Following equilibration of the sample to 45° C, 5  $\mu$ l of 1 U/ $\mu$ l  $\beta$ -agarase I (New England Biolabs, Inc.) was added, and the mixture was incubated for 90 minutes at 45° C to digest the agarose. After incubation, 40  $\mu$ l of 3 M Na acetate was added to the sample, and the mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose followed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories). The cDNA was ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 70  $\mu$ l water for the kinase reaction to phosphorylate the ligated Eco RI adapters.

To the 70  $\mu$ l cDNA solution was added 10  $\mu$ l 10x ligase buffer (Stratagene Cloning Systems), and the mixture was heated to 65° C for 5 minutes. The mixture was cooled on ice, and 16  $\mu$ l 10 mM ATP and 4  $\mu$ l of 10 U/ $\mu$ l T4 polynucleotide kinase (Stratagene Cloning Systems) were added. The reaction mixture was incubated at 37° C for 1 hour and was terminated by heating to 65° C for 10 minutes followed by serial extractions with phenol/chloroform and chloroform. The phosphorylated cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate, washed with 70% ethanol, air dried and resuspended in 10  $\mu$ l of water. The concentration of the phosphorylated cDNA was estimated to be -40 fmole/ $\mu$ l.

The pDX mammalian expression vector (disclosed in U.S. Patent No. 4,959,318) (Figure) was modified to accept 24-11-5#3 cDNA that had been synthesized with Eco RI-Xho I ends. An endogeneous Sal I site on pDX was eliminated by digesting the plasmid with Sal I and recircularizing the plasmid following blunting of the Sal I cohesive ends with T4 DNA polymerase. The recircularized plasmid was digested with Eco RI and to it was ligated a short polylinker sequence consisting of two complementary oligonucleotides, ZC6936 (SEQ ID NO: 15) and ZC6937 (SEQ ID NO: 16), to yield plasmid pDX.ES. The introduced polylinker sequence on pDX.ES contained Eco RI and Sal I sites to facilitate directional cloning of 24-11-5 cDNA synthesized with Eco RI-Xho I ends.

A plasmid cDNA library was prepared by ligating Eco RI-Xho I 24-11-5 cDNA into Eco RI/Sal I digested pDX.ES. The ligation mixture was electroporated into *E. coli* (ELECTROMAX DH10B™ competent cells; GIBCO BRL, Gaithersburg, MD) using a gene pulser/pulse controller and 0.2 cm cuvette (Bio-Rad Laboratories, Hercules, CA) employing a 0.2 KV, 400 ohm and 25  $\mu$ FAD. The cells were diluted to 1.5 ml in Luria broth and incubated at 37°C for 45 minutes followed by the addition of 0.75 ml of 50%

glycerol. The transfected cells were aliquotted and stored at -70°C until use. Eighty fmoles of cDNA gave rise to over 700,000 independent recombinant plasmids.

5 Example VII. Expression Screening of 24-11-5 cDNA Library for MPL Activity

The 24-11-5#3 cDNA library was plated onto approximately two thousand 10 cm diameter Luria broth agar plates supplemented with 100 µg/ml ampicillin. The  
10 plating density was between 200 and 250 bacterial colonies per plate. Plasmid DNA for transfection into BHK 570 cells was prepared from each bacterial plate using MAGIC MINIPREPS™ DNA purification resin (Promega Corp.), according to the manufacturer's instruction. Plasmid  
15 DNAs were stored at -20° C until transfection into BHK 570 cells.

Plasmid pools of 24-11-5#3 cDNA, each containing approximately 200 to 250 cDNA clones, were transfected into BHK 570 cells using a 3:1 liposome formulation of  
20 2,3-dioleoyloxy-N-[2(sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate and dioleoyl-phosphatidylethanolamine in water (LIPOFECTAMINE™; GIBCO BRL). Twenty µl of 30 ng/µl DNA was added to 20 µl of a 1:10 dilution of LIPOFECTAMINE™ solution and incubated at  
25 room temperature for 30 minutes. Following the incubation, 160 µl of serum-free media (Hams F12: Dulbeccos MEM (1:1) supplemented with 2 mM L-glutamine, 0.11 mg/ml sodium pyruvate, 5 µg/ml insulin, 5 µg/ml fetuin, 10 µg/ml transferrin, 2 ng/ml selenium IV oxide  
30 and 25 mM HEPES buffer) were added to the DNA/LIPOFECTAMINE™ mixture and transferred to a 24 well microtiter plate containing -100,000 BHK 570 cells. The cells were incubated at 37° C under 5% CO<sub>2</sub> for 4 hours, after which was added 200 µl of BHK Growth Media  
35 (Dulbecco's modified Eagles's media supplemented with 2 mM L-glutamine, 0.11 mg/ml sodium pyruvate, 5% heat

inactivated fetal calf serum and 100x PSN antibiotics (GIBCO BRL)). The cells were incubated for 16 hours. The media was removed and replaced with 0.5 ml of fresh BHK Growth Media, which was conditioned for 48 hours before  
5 being assayed for MPL activity.

A cell proliferation assay was used to detect the presence of MPL activity in conditioned media of library transfected BHK 570 cells. One hundred  $\mu$ l of conditioned media was added to 100  $\mu$ l of  $10^6$ /ml washed  
10 BaF3/MPLR1.1 cells in RPMI 1640 media (JRH Bioscience Inc., Lenexa, KS) supplemented with 2 mM L-glutamine, PSN antibiotics (GIBCO BRL), 0.00036% 2-mercaptoethanol and 10% heat inactivated fetal calf serum. The assay cells were incubated for 3 days at 37° C under 5% CO<sub>2</sub> before  
15 assaying for proliferation.

Cell proliferation in the presence of MPL was quantified using a colorimetric assay based on the metabolic breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosman, J. Immunol.  
20 Meth. 65: 55-63, 1983). Twenty  $\mu$ l of a 10 mg/ml solution of MTT (Polyscience, Inc., Warrington, PA) was added to 100  $\mu$ l of BaF3/MPLR1.1 assay cells, and the cells were incubated at 37° C. After 4 hours, 200  $\mu$ l of 0.04 N HCl in isopropanol was added, the solution was mixed, and the  
25 absorbance of the sample was read at 570 nm on a model EL320 ELISA reader (Bio-Tek Instruments Inc., Highland Park, VT).

One plasmid pool found to be positive, designated T1081, was transfected into BHK 570 cells.  
30 Supernatant from the transfectants gave a positive signal in the MTT proliferation assay. PCR and antibody neutralization experiments demonstrated that the activity was not due to IL-3 or IL-4.

Plasmids from the positive pool were used to  
35 transform E. coli DH10B, and cells were plated (42 plates with approximately 15-20 colonies per plate, 10 plates

with approximately 90 colonies per plate and 8 plates with approximately 250 colonies per plate). A replica of each plate was made and stored at 4°C. The colonies on the original plates were scraped and allowed to outgrow in liquid culture for several more hours, then DNA was prepared.

The plasmid DNA from the sub-pools was transfected into BHK 570 cells, and cell supernatants were collected and assayed as above. After approximately two hours, one sub-pool (#22) was scored as positive by microscopic examination (elongated cell shape). Several hours later two additional sub-pools (#19 and #28) were also scored positive. Remaining supernatants from each positive sub-pool were assayed against the control BaF3 cells and found to have no activity. In addition, the activity from the three positive sub-pools was found to be inhibited by the soluble Type I MPL receptor.

The replica plates from the three positive sub-pools were allowed to grow for several hours, then individual colonies were picked and used to inoculate 3-ml cultures. The cultures were grown approximately 8 hours at 37°C, then DNA was prepared by the miniprep method as described above. Plasmid DNA was transfected into BHK 570 cells, and supernatants were harvested approximately 10 hours later and assayed for activity. After one hour, one clone (designated T1081-19-215, corresponding to sub-pool #19) was scored positive. This clone was restreaked for single colonies. DNA was prepared from twelve colonies and transfected into BHK 570 cells. All twelve transfectants were later scored positive in the assay. DNA from one of the twelve positive colonies was transformed into *E. coli* DH5 $\alpha$ . The plasmid was designated pZGmpl-1081. This transformant has been deposited on February 14, 1994 with American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD under accession number 69566.

The nucleotide sequence of the cDNA encoding the hematopoietic protein (thrombopoietin) was determined (SEQ ID NO: 1). Analysis of the encoded amino acid sequence (SEQ ID NO: 2) indicated that the amino terminus of the  
5 mature protein is at amino acid residue 45. Two methionine codons, at positions 105 and 174 of SEQ ID NO: 1, appear to be initiation codons, with the major site of initiation expected to be at position 174.



Example VIII. Hematopoietic Activity of Recombinant Thrombopoietin

Marrow was harvested from femurs and tibias of a female CD-1 post-pregnant mouse into 25 ml of CATCH buffer (99 mg theophylline, 0.75 g sodium citrate, 75 mg adenosine, 20 ml of 10x Hank's balanced saline solution  $\text{Ca}^{++}$   $\text{Mg}^{++}$ -free, per 200 ml in  $\text{dH}_2\text{O}$ ; pH 7.4). Cells were suspended into single cell suspension by pipeting with a 25 ml pipet. The volume was brought up to 50 ml with CATCH buffer, and the cells were pelleted at 1000 rpm for 7 minutes. The pellet was resuspended in 25 ml CATCH buffer and incubated in a T75 tissue culture flask for a first round of plastic adherence at 37°C for 2 hours. Non-adherent cells were harvested by centrifugation at 1000 rpm for 7 minutes to pellet cells. The pellet was resuspended in 15 ml alpha-MEM + 10% FBS (+L-glutamine, NaPyruvate, and PSN antibiotics) and incubated in a T75 flask for a second round of plastic adherence as described above for the first round. Following the final centrifugation and resuspension, the cells were counted. One-half ml of cells at 576,000 cells/ml was plated into 24-well tissue culture plates, together with sample media from control BHK cells or with conditioned media from BHK cells transfected with pZGmpl-1081. After three days incubation at 37°C, the cells were harvested and stained as described below.

One hundred fifty  $\mu\text{l}$  of cells were harvested from the control well treated with standard conditioned medium. 50  $\mu\text{l}$  of cells were harvested from the well treated with conditioned medium from BHK cells transfected with pZGmpl-1081. These samples were spun, and standard microscope slides were prepared.

The slides were fixed in 100% methanol, then flooded with 1:1 Wright's (0.5 g Wright stain in 300 ml methanol)/ $\text{H}_2\text{O}$  for 6 minutes, washed with water, and

dried. Slides were then flooded with Giemsa stain (Sigma Chemical Corp.) in Sorensen buffer (2.28 g  $\text{KH}_2\text{PO}_4$ /2.38 g  $\text{NaPO}_4$  in 250 ml  $\text{H}_2\text{O}$ ), washed with water, and dried.

After adjusting for the volumes used, the  
5 BHK/pZGmpl-1081 medium sample contained 120 megakaryocytes per 150  $\mu\text{l}$  volume as compared to 9 megakaryocytes per 150  $\mu\text{l}$  volume of control medium. In addition, the megakaryocytes in the treated experimental sample were  
10 observed microscopically to be significantly larger in size than control cells and to have significantly higher staining for polynuclei content.

Conditioned media from the mutant BaF3/MPLR1.1 line 24-11-5 #3 was collected in the absence of serum and concentrated 20-fold on a 10Kd cut-off Amicon Inc.  
15 (Beverly, MA) filtration device. Marrow was harvested from mouse femurs and suspended in Iscove's Modified Dulbecco's Media (GIBCO BRL) + 15% fetal calf serum (FCS). Following suspension, nucleated cells were counted and plated at 75,000 cells/ml with 0.9 ml/plate in medium  
20 adjusted to contain 50% methylcellulose, 15% FCS, 10% BSA, and 0.6% PSN (semi-solid medium) in 1 ml tissue culture plates. Various conditioned medium and control samples were added to bring the total volume to 1 ml. Plates were incubated at 37°C/5%  $\text{CO}_2$  for 6 days and then examined  
25 microscopically for counts of granulocyte/macrophage (GM) colonies. Plates incubated in the presence of the 24-11-5 #3 conditioned medium were observed to have weak GMCSF-like activity, producing a colony count of 25, compared with a count of zero for the negative control sample, and  
30 a count of 130 for a plate stimulated with a positive control (pokeweed mitogen spleen conditioned medium (PWMSM); prepared by incubating minced mouse spleen for one week in the presence of pokeweed mitogen (obtained from Boehringer Mannheim, Indianapolis, IN) + 2 units/ml  
35 erythropoietin)

Marrow was harvested from mouse femurs and suspended in Iscove's Modified Dulbecco's Media (GIBCO-BRL) containing 15% FCS, and nucleated cells were counted and plated in semi-solid medium as described above. The  
 5 cells were used to test megakaryocyte colony forming activity of the protein encoded by the pZGmpl-1081 insert.

A pool of BHK 570 cells stably transfected with pZGmpl-1081 was cultured in the absence of serum, and conditioned medium was collected. The conditioned medium  
 10 was tested alone and in combination with pokeweed mitogen spleen conditioned medium, recombinant mouse IL-3, IL-6 (Genzyme Corp., Cambridge, MA), IL-11 (Genzyme Corp.) or combinations of these factors. PWMSCM was used as a positive control. Non-conditioned culture medium was used  
 15 as a negative control.

Test or control samples were added to the marrow cultures to bring the total volume to 1 ml. The plates were incubated for six days at 37°C in 5% CO<sub>2</sub>, then examined microscopically for counts of megakaryocyte  
 20 colonies. Results are shown in Table 4. To summarize, the BHK/pZGmpl-1081 conditioned medium exhibited megakaryocyte colony forming activity, which was enhanced in the presence of early-acting factors to levels notably higher than any of the early-acting factors alone.

25

Table 4

		Megakaryocyte
<u>Sample</u>		<u>Colonies</u>
30	Negative control	0
	PWMSCM	7
	BHK/pZGmpl-1081	2
	BHK/pZGmpl-1081 + PWMSCM	15
	IL-3	1
35	IL-3 + BHK/pZGmpl-1081	8
	IL-6	0
	IL-6 + BHK/pZGmpl-1081	6

Table 4 continued

	IL-11	1
	IL-11 + BHK/pZGmpl-1081	6
	IL-3 + IL-6	2
5	IL-3 + IL-6 + BHK/pZGmpl-1081	9
	IL-3 + IL-11	5
	IL-3 + IL-11 + BHK/pZGmpl-1081	15

10        *In vivo* activity of the BHK/pZGmpl-1081  
 conditioned medium was assayed in mice. Serum-free medium  
 was collected and concentrated five-fold using a 10 Kd  
 cutoff filtration device (Amicon, Inc., Beverly, MA).  
 Control (non-conditioned) medium was concentrated in a  
 like manner. Six BALB/c mice (Simonsen Laboratories,  
 15        Inc., Gilroy, CA) were treated with seven daily  
 intraperitoneal injections of 0.5 ml of either the control  
 or conditioned medium. Blood samples were collected on  
 days 0, 3, and 7 and counted for platelet content.  
 Results, shown in Table 5, demonstrate that the  
 20        conditioned medium from BHK/pZGmpl-1081 cells has  
 thrombopoietic activity.

Table 5

		Platelet count ( $10^4/\mu\text{l}$ )		
25	<u>Treatment</u>	<u>Day 0</u>	<u>Day 3</u>	<u>Day 7</u>
	Control	141	141	87
	Control	159	149	184
	BHK/pZGmpl-1081	157	160	563
	BHK/pZGmpl-1081	169	154	669
30	BHK/pZGmpl-1081	139	136	492
	BHK/pZGmpl-1081	135	187	554

#### Example IX. Isolation of Human Thrombopoietin Gene

35        An amplified human lung Lambda FIX<sup>®</sup> genomic  
 library (Stratagene Cloning Systems) was screened for the

gene encoding human thrombopoietin using the mouse *mpl* receptor ligand cDNA as a probe. The library was titrated, and 30 150-mm plates inoculated with *E. coli* strain LE-392 cells (Stratagene Cloning Systems) were infected with 4 x 10<sup>4</sup> plaque forming units (PFU). The plates were incubated overnight at 37°C. Filter plaque lifts were made using HYBOND-N<sup>TM</sup> nylon membranes (Amersham) according to the procedure recommended by the manufacturer. The filters were processed by denaturation in a solution containing 1.5 M NaCl and 0.5 M NaOH for 7 minutes at room temperature. The filters were blotted briefly on filter paper to remove excess denaturation solution followed by neutralization for 5 minutes in 1 M Tris-HCl (pH 7.5) and 1.5 M NaCl. Phage DNA was fixed onto the filters with 1,200  $\mu$ Joules of UV energy in a STRATALINKER<sup>®</sup> UV crosslinker (Stratagene Cloning Systems). After fixing, the filters were prewashed three times in 0.25 x SSC, 0.25% SDS and 1 mM EDTA at 65°C. After prewashing, the filters were prehybridized in hybridization solution (5x SSC, 5X Denhardt's solution, 0.2% SDS and 1 mM EDTA) that had been filtered through a 0.45  $\mu$ M filter. Heat denatured, sheared salmon sperm DNA (final concentration 100  $\mu$ g/mL) was added immediately before use. The filters were prehybridized at 65°C overnight.

Full length mouse TPO cDNA from pZGmpl-1081 was labeled with <sup>32</sup>P by random priming using the MEGAPRIME<sup>TM</sup> DNA Labeling System (Amersham) according to the method recommended by the manufacturer. The prehybridization solution was replaced with fresh hybridization solution containing approximately 1 x 10<sup>6</sup> cpm probe and allowed to hybridize overnight at 65°C. After hybridization, the hybridization solution was removed, and the filters were rinsed four or five times each in a wash solution containing 0.25x SSC, 0.25% SDS, and 1 mM EDTA. After rinsing, the filters were washed in eight consecutive washes at 50°C in wash solution. Following the final wash,

the filters were exposed to autoradiograph film (XAR-5; Eastman Kodak Co.; Rochester, NY) for four days at -70°C with an intensifying screen.

Examination of the autoradiographs revealed several hundred regions that hybridized with the labeled probe. Agar plugs were picked from 100 regions for purification. Each agar plug was soaked overnight in 1 ml of SM containing 1% (v/v) chloroform (Maniatis et al., eds., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1982). After the overnight incubation, the phage from each plug were diluted 1:1,000 in SM. Aliquots of 5 µl were plated on *E. coli* strain LE392 cells. The plates were incubated overnight at 37°C, and filter lifts were prepared, prehybridized, hybridized, washed and autoradiographed as described above.

Examination of the resulting autoradiographs revealed strong positive signals from two primary isolates and weak signals from eighteen others. Agar plugs were picked from the positive areas for each of the twenty signals. The agar plugs were treated as described above. The phage eluted from each agar plug were diluted 1:100 in SM, and aliquots of 1 µl were plated with *E. coli* strain LE392 cells. The plates were incubated, and phage filter lifts were prepared and hybridized as described above. The filters were washed at 55°C in wash buffer. Autoradiographs of the filters revealed areas of hybridization corresponding to single, discrete phage plaques from three original isolates, 8-3-2, 10-1-1 and 29-2-1.

Phage isolates 8-3-2, 10-1-1 and 29-2-1 were given the designations λZGmpl-H8, λZGmpl-H10 and λZGmpl-H29, respectively. DNA from isolates λZGmpl-H8, λZGmpl-H10 and λZGmpl-H29 was purified using LAMBDA SORB™ phage adsorbent (Promega Corp., Madison, WI) according to the directions of the manufacturer. Human genomic DNA inserts from the phage were separated from phage vector DNA by

digestion with Xba I and purified by agarose gel electrophoresis. All three phage isolates contained sequences which hybridized to the mouse *mpl* receptor ligand cDNA probe as shown by Southern blot analysis (Maniatis et al., *ibid*). Phage  $\lambda$ ZGmpl-H8 was analyzed and the hybridizing regions of  $\lambda$ ZGmpl-H8 were found to reside on three Xba I DNA fragments of 9.5 kb, 2.5 kb and 1 kb in length. The 2.5 kb fragment was subcloned into Xba I digested BLUESCRIPT® II SK+ phagemid (Stratagene Cloning Systems), to yield the plasmid pZGmpl-H82.5.

The sequence of the human TPO gene and the encoded amino acid sequence are shown in SEQ ID NO: 28 and SEQ ID NO: 29.

Example X. Isolation of Full-length Human Thrombopoietin cDNA.

A full-length human TPO encoding cDNA was isolated by polymerase chain reaction from human liver and kidney cDNA templates employing specific primers derived from exon sequences identified on pZGmpl-H82.5 and from conserved 5' untranslated sequence of the mouse TPO cDNA.

Human kidney, liver and lung poly d(T) selected poly(A)<sup>+</sup> RNAs (Clontech, Palo Alto, CA) were used to synthesize first strand cDNA. Each reaction was prepared using four micrograms poly(A)<sup>+</sup> RNA mixed with 1  $\mu$ g of oligo d(T)<sub>18</sub> (No 5' Phosphate) mRNA primer (New England Biolab, Beverly, MA) in a final volume of 19  $\mu$ l. The mixtures were heated to 65°C for five minutes and cooled by chilling on ice. cDNA synthesis was initiated by the addition of 8  $\mu$ l of 5x SUPERSCRIPT™ buffer (GIBCO BRL), 2  $\mu$ l of 100 mM dithiothreitol, 2  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), 2  $\mu$ l of 1  $\mu$ Ci/ $\mu$ l <sup>32</sup>P-  $\alpha$ -dCTP (Amersham, Arlington Heights, IL) and 8  $\mu$ l of 200 U/ $\mu$ l SUPERSCRIPT™ reverse transcriptase (GIBCO BRL) to each of the RNA-primer mixtures. The

reactions were incubated at 45°C for 1 hour and were diluted to 120 µl with TE (10 mM Tris:HCl, pH 8.0, 1 mM EDTA). The cDNAs were precipitated twice by the addition of 50 µl 8 M ammonium acetate and 160 µl of isopropanol. 5 The resulting cDNA pellets were resuspended in 10 µl of TE. The yield of first strand cDNA for each reaction was estimated from the levels of <sup>32</sup>P-dCTP incorporation.

First strand cDNA from the liver, lung and kidney mRNA were used to generate two cDNA segments, an N-terminal one third and the C-terminal two thirds of the 10 sequence, using separate polymerase chain reactions. A Kpn I restriction site was introduced into the cDNA segments by a single base change from the genomic sequence by PCR mutagenesis employing primers ZC7422 (SEQ ID NO: 20) and ZC7423 (SEQ ID NO: 21). 15 The resulting nucleotide change created a common KpnI restriction site without alteration in the predicted amino acid coding.

The N-terminal segment was amplified in a 50 µl reaction containing 5 ng of template cDNA (in separate 20 reactions for kidney, liver and lung cDNAs), 80 pmoles each of oligonucleotides ZC7424 (SEQ ID NO: 22) and ZC7422 (SEQ ID NO: 20), 5 µl of 2.5 mM deoxynucleotide triphosphate solution (Cetus Corp., Emeryville, CA), 5 µl of 10x PCR buffer (Promega Corp., Madison, WI) and 2.5 25 units of Taq polymerase (Boehringer Mannheim). The polymerase chain reaction was run for 35 cycles (1 minute at 94°C, 1 minute at 58°C and 1.5 minute at 72°C) followed by a 7 minute incubation at 72°C. Sense primer ZC7424 (SEQ ID NO:22) spanned the mouse *mpl* receptor ligand 5' 30 nontranslated region and include the ATG initiation codon. Antisense primer ZC7422 (SEQ ID NO:20) included sequence from the region corresponding to exons 4 and 5 of the human genomic TPO DNA.

The C-terminal segment was amplified in a 50 µl 35 reaction containing 5 ng of template cDNA (human kidney, liver or lung as described above), 80 pmoles each of



oligonucleotides ZC7423 (SEQ ID NO:21) and ZC7421 (SEQ ID NO:23), 5  $\mu$ l of 2.5 mM deoxynucleotide triphosphate solution (Cetus Corp.), 5  $\mu$ l of 10X PCR buffer (Promega Corp.) and 2.5 units of Taq polymerase (Boehringer Mannheim). The polymerase chain reaction was run for 35 cycles (1 minute at 94°C, 1 minute at 65°C and 1.5 minutes at 72°C) followed by a 7 minute incubation at 72°C. Sense primer ZC7423 (SEQ ID NO: 21) included sequence from regions corresponding to exons 4 and 5 of the human genomic TPO DNA. Antisense primer ZC7421 (SEQ ID NO:23) included sequence from the region corresponding to the 3' noncoding sequence of the human gene and included the translation termination codon.

The amplified PCR products were analyzed by direct DNA sequencing and were subcloned into pGEM-T (Promega Corp.) for further analysis by comparison to the mouse cDNA sequence and to human genomic sequences. A DNA sequence encoding human TPO is shown in SEQ ID NO: 18, and the encoded amino acid sequence is shown in SEQ ID NO: 19. Sequence analysis indicates that signal peptide cleavage occurs at amino acid 22 (SEQ ID NO: 19) and the mature protein begins at amino acid 22 (SEQ ID NO: 19).

The human N-terminal and C-terminal PCR fragments were excised from pGEM-T as EcoRI-KpnI fragments and ligated into the EcoRI site of expression vector Zem229R. This plasmid was transfected into BHK 570 cells using Lipofectamine™ (GIBCO BRL). 24 hours after transfection, the culture medium (DMEM + PSN + 10% FCS) was replaced with fresh medium, and the cells were incubated for 48 hours in the absence of selective agents. Conditioned medium was assayed for proliferative activity using the BaF3/MPLR1.1 cell line as described previously. The results clearly showed that the human TPO in the culture medium stimulated the proliferation of the BaF3 cells expressing the mouse MPL receptor.

CDNA was made from both human liver and kidney mRNA (obtained from Clontech Laboratories, Inc.) using SUPERScript™ reverse transcriptase (GIBCO BRL) according to the manufacturer's specifications. Liver- and kidney-  
5 derived human TPO DNA clones were then made using two PCR reactions (conditions shown in Table 6). The reactions were run for 35 cycles at 94° C for 1 minute, 58° C for 1 minute, 72° C for 1.5 minute; followed by a 7 minute incubation at 72° C.

10

Table 6

## Reaction #1:

5 ng liver or kidney cDNA  
4 µl oligonucleotide ZC7454 (20 pM/µl) (SEQ ID NO:24;  
15 introduces an EcoRI site 5' of the ATG)  
4 µl oligonucleotide ZC7422 (20 pM/µl) (SEQ ID NO:20;  
creates an Asp718 site)  
5 µl dNTPs solution containing 2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dCTP and 2.5 mM dTTP  
20 5 µl 10X Taq buffer (Boehringer Mannheim)  
1 µl Taq polymerase (Boehringer Mannheim)  
30 µl H<sub>2</sub>O

## Reaction #2:

25 5 ng liver or kidney cDNA  
4 µl oligonucleotide ZC7423 (20 pM/µl) (SEQ ID NO:20;  
creates an Asp718 site)  
4 µl oligonucleotide ZC7453 (20 pM/µl) (SEQ ID NO:25;  
creates an EcoRI site 3' of the TGA)  
30 5 µl dNTPs solution containing 2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dCTP and 2.5 mM dTTP  
5 µl 10X Taq buffer (Boehringer Mannheim)  
1 µl Taq polymerase (Boehringer Mannheim)  
35 30 µl H<sub>2</sub>O

The PCR products were treated with phenol/chloroform/isoamyl alcohol and precipitated with 95% ETOH, dried, and resuspended in 20  $\mu$ l H<sub>2</sub>O. Each product was then cut with the restriction enzymes Asp718 and EcoRI and electrophoresed on a 1% agarose gel. 410 bp fragments (liver and kidney) from Reaction #1 and 699 bp fragments (liver and kidney) from Reaction #2 were excised from the gel and eluted by centrifugation of gel slabs through nylon wool. The PCR products of Reaction #1 and Reaction #2 were ligated together with the vector Zem229R (deposited with American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD on September 28, 1993 under accession number 69447) which had been cut with EcoRI, thereby joining the two products at a created Asp718 site. The resultant plasmids were designated #10 (containing the kidney derived cDNA) and #28 (containing the liver derived cDNA).

Upon sequencing the DNAs, single PCR-generated errors were found 5' and 3' of a unique AvrII site in the #28 and #10 plasmids, respectively. To create an error-free TPO DNA, an 826 bp EcoRI-AvrII 5' fragment was isolated from #10 and a 283 bp AvrII-EcoRI 3' fragment was isolated from #28. The two fragments were ligated together with the vector Zem229R which had been cut with EcoRI. The resultant plasmid was designated pZGmpl-124. This plasmid was deposited with American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD on May 4, 1994 as an *E. coli* DH10b transformant under accession number 69615.

30

#### Example XI. Megakaryocyte cDNA Library

To amplify megakaryocyte precursors in vivo 20 mice were injected interperitoneally with 40,000 activity units (units being defined as 50 U/ml to obtain one-half maximal proliferation rate of BaF3/MPLR1.1 cells in the MTT assay (Example VII)) of recombinant murine

35

thrombopoietin daily (concentrated serum-free conditioned media from BHK 570 cells stably transfected with mouse thrombopoietin cDNA). On the fifth day of injections, spleens were removed and placed into CATCH buffer + Hepes (Hank's balanced salt solution (HBSS) calcium and magnesium free, 10 mM Hepes (GIBCO BRL), 1.4 mM adenosine, 2.74 mM theophylline (Sigma Chemical Co., St. Louis, MO) and 0.38% sodium citrate (J.T. Baker Inc., Philipsburg, NJ) pH adjusted to 7.40 with sodium hydroxide). Five spleens were processed at a time by making an incision in each and milking out cells between two stainless steel meshes into CATCH buffer + Hepes. After breaking apart some of the cell clumps with a 25 ml pipette the volume was increased to 50 ml, and cells were spun down for 7 minutes at 208 x g in a Sorval TJ-6 centrifuge. Each cell pellet was resuspended in 10 ml of CATCH buffer + Hepes and filtered through 130  $\mu$ m nylon mesh to obtain single-cell suspensions. The volumes were increased to 50 ml with CATCH buffer + Hepes, and cells were spun down for 15 minutes at 33 x g. The cells were washed with an additional 50 ml of CATCH + Hepes and spun for 10 minutes at 33 x g. The cell pellets were resuspended in 10 ml of CATCH buffer + Hepes and layered onto a three-step Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) gradient (65, 40 and 27% in 1X CATCH buffer + Hepes, 12 ml each in a 50 ml centrifuge tube) and centrifuged for 45 minutes at 833 x g. Cells between the 40 and 63% Percoll layers were collected, and the volumes were increased to 50 ml with CATCH buffer + Hepes. Cells were spun down for 7 minutes at 208 x g and resuspended in 50 ml of megakaryocyte growth media (minimal essential medium alpha modification, ribonucleoside- and deoxyribonucleoside-free with 15% heat inactivated fetal bovine serum, 2 mM L-glutamate (media components obtained from JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate (Irvine Scientific, Santa Ana, CA), 1 X PSN antibiotic mixture (GIBCO BRL)) and 1,000 activity

units of recombinant murine thrombopoietin/ml added (serum-free conditioned media from BHK 570 cells stably transfected with the mouse thrombopoietin cDNA). Cells were then plated on 150 mm tissue culture dishes at  $10^6$  mononucleated cells/ml and grown in a fully humidified incubator with 6.0% CO<sub>2</sub> in air at 37°C. After three days of growth nonadherent cells were collected in 50 ml centrifuge tubes and cooled on ice. Large cells were pelleted by centrifuging at 33 x g for 15 minutes at 4°C. Cell pellets were resuspended in 50 ml CATCH buffer + Hepes at room temperature and spun down for 10 minutes at 33 x g. (All further steps were performed at room temperature.) This wash was repeated again to obtain a higher purity of mature megakaryocytes. The remaining cells were resuspended in 15 ml of CATCH + Hepes (pooled volume) and layered onto three fetal bovine serum step gradients (JRH Biosciences) (65% and 40% diluted with CATCH buffer + Hepes) for sedimentation at 1 x g for 30 minutes. The bottom 5 ml of the 65% fractions were pooled, diluted to 50 ml with CATCH buffer + Hepes, and spun down for 10 minutes at 33 x g. The pellet contained more than  $10^7$  cells. The cells were assayed for acetylcholinesterase by the method of Burstein et al. (J. Cell. Physiol. 122: 159-165, 1985) and determined to be mature megakaryocytic cells with purity of greater than 99%. The pelleted cells were then lysed in guanidium thiocyanate/2-mercaptoethanol solution for RNA isolation by cesium chloride density gradient centrifugation.

cDNA is prepared from the megakaryocyte RNA as disclosed in Example VI, above.

Example XII. Fluorescence in situ Hybridization Mapping of the Human Thrombopoietin Gene

The following were added to 1.5 ml microcentrifuge tubes on ice: 1 µg λZGmpl-H8, λZGmpl-H10 or

λZGmpl-H29 containing the human thrombopoietin gene, 5 μl  
10 x nick translation buffer (0.5 M Tris/HCl, 50 mM MgCl<sub>2</sub>,  
0.5 mg/ml BSA (nuclease free)), 5 μl dNTPs solution  
containing 0.5 mM dATP, 0.5 mM dGTP and 0.5 mM dCTP, 5 μl  
5 5 mM Bio-11-dUTP (5-(N-[N-biotinyl-ε-aminocaproyl]-3-amino-  
allyl)-2'-deoxyuridine 5'-triphosphate, Sigma Chemical  
Co.), 5 μl 100 mM DTT, 5 μl DNase I (a 1000 x dilution  
from a 10 U/μl stock, Boehringer Mannheim, RNase-free),  
2.5 μl DNA polymerase I (5 U/μl, Boehringer Mannheim), H<sub>2</sub>O  
10 to a final volume of 50 μl. After mixing, the reactions  
were incubated at 15°C for 2 hours in a Boekel mi-  
crocooler. The reactions were stopped by adding 5 μl 0.5  
M EDTA, pH 7.4 to the reactions. The probes were purified  
using Sephadex® G-50 DNA purification spin columns  
15 (Worthington Biochemical Corporation, Freehold, NJ)  
according to the manufacturer's instructions. To check  
the size of the labeled probes, 5 - 10 μl of each purified  
probe was mixed with 5 μl gel loading buffer (12.5%  
ficoll, 0.2% bromphenol blue, 0.2 M Tris-acetate, 0.1 M  
20 sodium acetate, 1 mM EDTA) and run out on a 0.7% agarose  
mini-gel at 80 V. λ-Hind III fragments (GIBCO BRL) and φX-  
Hae III fragments (GIBCO BRL) were used as base pair (bp)  
size markers. A digoxigenin-labeled centromeric probe  
specific to chromosome 3 (D3Z1) was obtained from Oncor  
25 (Gaithersburg, MD).

Metaphase chromosomes were obtained from a HEL  
cell culture. 100 μl Colcemid® (GIBCO BRL, 10 μg/ml stock)  
was added to the media of the 100 x 15 mm petri dish used  
for the cell culture and incubated at 37°C. After 2.5 - 3  
30 hours, the media was removed from the petri dish using a  
10 ml sterile plastic pipette and transferred to a 15 ml  
polypropylene conical tube (Blue Max™, Becton Dickinson).  
2 ml of 1 x PBS (140 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5  
mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) was added to the petri dish for rinsing  
35 using a 5 ml sterile plastic pipette and transferred to  
the conical tube. 2 ml of trypsin (GIBCO BRL, stock

solution) was added to the petri dish using a sterile 5 ml plastic pipette, and the petri dish was gently rocked and put into a 37°C incubator for 3-5 minutes. The cells were then washed from the petri dish using a 5 ml sterile plastic pipette and added to the tube with the media. The culture tube was centrifuged at 250 x g for 8 minutes, and all but 0.5 ml of the supernatant was removed. The pellet was resuspended by tapping, then slowly and gently 8 ml of 0.075 M KCl (prewarmed to 37°C) was added. The suspension was mixed gently and placed in a 37°C water bath for 10 minutes. The solution was centrifuged at 250 x g for 5 minutes, and all but 0.5 ml of the supernatant above the pellet was removed. The pellet was resuspended by tapping the tube. Two ml of cold methanol:acetic acid (3:1) was added dropwise with shaking to fix the cells. A total of 8 ml of fix was added in this manner. The tube was placed in the refrigerator for 20 minutes, followed by a 5 minute centrifugation at 250 x g. The supernatant was again aspirated off and the fixation process repeated two more times. To drop metaphase spreads on 25 x 75 mm precleaned, frosted glass slides (VWR Scientific, Media, PA), 5 µl of 50% acetic acid was spotted on each slide with a 20 µl Pipetman™ (Gilson Medical Electronics, Inc., Middleton, WI), followed by 5 µl of the cell suspension. The slides were allowed to air dry and then aged overnight in a 42°C oven (Boekel Industries, Inc., Philadelphia, PA) before use. The slides were scored for suitable metaphase spreads using a microscope equipped with a phase contrast condenser. Some metaphase chromosome preparations were G-banded with Gurr's improved R66 Giemsa's stain (BDH Ltd., Dorset, England), photographed, and destained before being used for the hybridization experiments. Slide preparations with human metaphase chromosome spreads were incubated for 2 hours in 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), rinsed briefly in H<sub>2</sub>O and stained in Gurr's Giemsa's stain which had been diluted 1:4 in

Giemsa's buffer solution, pH 6.5 (BDH Ltd.) and filtered through a Whatman #1 filter before use. Some preparations were incubated first for 45 minutes to 1 hour in a 90°C oven and allowed to cool before incubation in SSC. The  
5 preparations were then differentiated in Giemsa's buffer solution, rinsed in H<sub>2</sub>O and air dried. Suitable G-banded metaphase chromosome spreads were photographed on an Olympus microscope using Kodak Ektachrome™ 400 slide film and digitized and stored using an Optronics (Goleta, CA)  
10 ZVS-47E CCD RGB color video camera system and Optimus software (from BioScan Inc., Edmonds, WA). Preparations were destained for about 20 min. in 100% EtOH and air dried before further use. Unused metaphase chromosome slide preparations were stored at -70°C.

15 Hybridization mixes were prepared in 1.5 ml sterile microcentrifuge tubes by combining 2.5 µg competitor DNA (Cot-1 DNA, GIBCO BRL), 40-60 ng biotin-labeled λZGmpl-H8, λZGmpl-H10 or λZGmpl-H29 phage (containing the human thrombopoietin gene), 7 µg carrier DNA  
20 (denatured salmon testes DNA, Sigma Chemical Co.), 1 ml 3 M NaOAc and 2 volumes ethanol were vacuum dried in a speedvac concentrator. The pellet was dissolved in 10 µl of a hybridization solution consisting of 10% dextran sulfate, 2 x SSC and 50% formamide (EM Science, Gibbstown, NJ). The probe and competitor DNA were denatured at 70 -  
25 80°C for 5 minutes, chilled on ice and preannealed at 37°C for 1-2 hours. Denaturation of the chromosomes was done by immersion of each slide in 70% formamide, 2 x SSC at 70-80°C for 5 minutes, followed by immediate cooling in  
30 ice-cold 70% ethanol, then in 100% ethanol for 5 - 10 minutes each. The slides were then air dried and warmed to 42°C just before pipeting the hybridization mixtures onto them with a 20 µl Gilson Pipetman™. The hybridization mixtures and chromosomes were then covered with 18 x 18  
35 mm, No. 1 coverslips (VWR Scientific). The hybridizations proceeded in a moist chamber overnight at 37°C. In some



cases, after approximately 6 hours of hybridization time,  
5 - 10 ng of denatured, digoxigenin-labeled D3Z1  
centromeric probe (in 10% dextran sulfate, 2 x SSC and 65%  
formamide hybridization solution) was added to  
5 preparations.

After removal of the coverslips, the slides were  
washed 3 x 5 minutes in 50% formamide, 2 x SSC at 42°C, 3  
x 5 minutes in 2 x SSC at 42°C and 1 x 3 minutes in 4 x  
SSC, 0.05% polyoxyethylenesorbitan monolaurate (Tween-20,  
10 Sigma Chemical Co.). This was followed by a 20 minute  
preincubation with 4 x SSC containing 5% non-fat dry milk  
in a moist chamber (100 µl under a 24 x 50 mm coverslip).  
For the preparations that included the chromosome 3 D3Z1  
centromeric probe, a 45 minute incubation was then carried  
15 out with a 1:100 dilution of biotin-labeled, mouse anti-  
digoxin (Sigma Chemical Co.) in 4 x SSC/5% BSA, followed  
by three 3-minute washes in 4 x SSC, 0.05% Tween-20. The  
post-hybridization steps then proceeded for all prepa-  
rations, with a 20 minute incubation with fluorescein-  
20 labeled avidin (Flourescein Avidin DCS, Vector  
Laboratories, Burlingame, CA) (100 µl, 5 µg/ml, in 4 x  
SSC, 5% non-fat dry milk) under a 24 x 50 mm coverslip.  
The slides were then washed 3 x 3 minutes in 4 x SSC,  
0.05% Tween-20, followed by a 20 minute incubation with  
25 biotinylated goat anti-avidin D (affinity purified, Vector  
Laboratories) (5µg/ml in 4 x SSC, 5% non-fat dry milk)  
under a 24 x 50 mm coverslip. The slides were washed  
again 3 x 3 minutes in 4 x SSC, 0.05% Tween 20, followed  
by another incubation with fluorescein-labeled avidin (100  
30 µl/ml in 4 x SSC, 5% non-fat dry milk) under a 24 x 50 mm  
coverslip. In some cases, the signal amplification  
procedure was repeated one additional time. The final  
washes were for 2 x 3 minutes in 4 x SSC, 0.05% Tween-20  
and 1 x 3 minutes in 1 x PBS. The slides were mounted in  
35 antifade medium consisting of 9 parts glycerol containing  
2% 1,4-diazobicyclo-(2,2,2)-octane (DABCO, dissolved at

70°C) and one part 0.2 M Tris/HCl, pH 7.5 and 0.25-0.5 µg/ml propidium iodide. The slides were viewed on an Olympus BH2 microscope equipped with a BH2-RFC reflected light fluorescence attachment, a PM-10 ADS automatic photomicrographic system, an Optronics ZVS-47E CCD RGB color video camera system and a Chroma Technology Corp. (Brattlebow, VT) FITC/Texas Red filter set for FITC visualization. Images of the metaphase chromosome spreads were digitized and stored using an Optronics video imaging camera system and Optimus software.

The preliminary results from the physical mapping procedure indicated that the human thrombopoietin gene locus is distal on the q arm of chromosome 3 in the 3q26 region.

Example XIII. Expression of Mouse TPO Cytokine Domain in *Saccharomyces cerevisiae*

Plasmid pBJ3-5 contains the *S. cerevisiae* *TPI1* promoter, the  $\alpha$ -factor secretion leader, the mouse TPO coding sequence (SEQ ID NO: 1) from bp 237 to 692, the *TPI1* transcription terminator, 2µ sequences for replication in yeast and the *Schizosaccharomyces pombe* triose phosphate isomerase gene (*POT1* gene) for selection in yeast. This plasmid was designed to direct secretion of a mouse TPO protein containing amino acids 45-196 of SEQ ID NO: 2.

To construct pBJ3-5, pMVR1 (Figure 2) was digested with SphI and XbaI, and the vector backbone containing the 5' part of the *TPI1* promoter and the *TPI1* terminator was recovered. The following fragments were then inserted into the vector backbone:

- 1) An SphI/HindIII fragment derived from pBS114 which contains the 3' part of the *TPI1* promoter and the  $\alpha$ -factor leader. Plasmid pBS114 is a yeast shuttle vector that contains the *TPI1* promoter and the  $\alpha$ -

factor leader followed by a polylinker sequence which includes a HindIII site.

2) A PCR-generated HindIII/SalI fragment containing a HindIII site designed to be in-frame with the HindIII site in the  $\alpha$ -factor leader, a Kex2 proteolytic cleavage site and the mouse TPO sequence from bp 237 to 335 of SEQ ID NO: 1.

3) A SalI/EcoRI fragment containing mouse TPO base pairs 336 to 692 of SEQ ID NO: 1 which was derived from plasmid pSL-MPL-100 (constructed by amplifying pZGmpl-1081 using primers ZC7319 (SEQ ID NO: 27) and ZC7318 (SEQ ID NO: 26), digesting with Eco RI and cloning the fragment comprising TPO cytokine domain sequence and 5' non-coding sequence into the Eco RI site of Zem229R [ATCC 69447]). This fragment was changed to a SalI/XbaI fragment by cloning it into pIC19H which was first digested with SalI and EcoRI.

The resulting plasmid, designated pBJ3 (Figure 2), was then digested with BglII and XhoI to liberate the entire expression cassette containing the promoter, leader, TPO coding sequence and terminator. This BglII/XhoI fragment was inserted into pRPOT (disclosed in U.S. Patent No. 5,128,321; which is incorporated herein by reference) which had been digested with BamHI and XhoI. The resulting plasmid was designated pBJ3-5.

*S. cerevisiae* strain JG134 (*MAT $\alpha$*  *ura3-52 leu2-A2 pep4- $\Delta$ 1 Atp1::URA3 [*cir*<sup>0</sup>]) was transformed with pBJ3-5 and pRPOT by the lithium acetate procedure (as generally disclosed by Ito et al., *J. Bacteriol.* 153: 163-168, 1983). Transformants were selected by their growth on glucose-containing media. JG134/pBJ3-5 and JG134/pRPOT were grown in YEPD liquid media for three days. Culture*

media were separated from the cells by centrifugation and analyzed by the cell proliferation assay in BaF3 cells containing the MPL receptor. Media from strain JG134/pBJ3-5 contained 5000-7000 units/ml of TPO activity while the negative control JG134/pRPOT had no activity. This result indicates that yeast can secrete a biologically active form of TPO.

Example XIV. Activity of Recombinant Human TPO

Plasmid DNA from two 5 ml overnight bacterial cultures transformed with pZGmpl-124 was prepared by alkaline cell lysis followed by binding of DNA to a resin at high salt (using a Magic Minipreps™ Sampler kit from Promega Corp.). The DNA was eluted with 75 µl 10 mM Tris, 1 mM EDTA, pH 8.0.

BHK 570 cell cultures at 50,000 cells/well were transfected with pZGmpl-124 DNA. 20 µl of a 1:10 dilution of LIPOFECTAMINE™ (GIBCO BRL) was added to 20 µl of plasmid DNA and 160 µl of serum free media (F/DV media [a 1:1 mixture of DMEM and Ham's F12] supplemented with 10 µg/ml fetuin, 2 ng/ml selenium, 5 µg/ml insulin, 10 µg/ml transferin, 2 mM L-glutamine, 110 µg/ml sodium pyruvate, 25 mM HEPES, and 0.1 mM non-essential amino acid solution (GIBCO BRL)) for 30 minutes at room temperature before adding to BHK 570 cells and incubating for 4 hours at 37°C. 200 µl of Growth Media (DMEM (Biowhittaker) supplemented with 2 mM L-glutamine, 110 µg/ml sodium pyruvate, 0.05 mg/ml penicillin, 0.05 mg/ml streptomycin, 0.01 mg/ml neomycin, 25mM HEPES, 10% fetal calf serum) was then added, and the cells were incubated at 37°C overnight. The culture media was then replaced with Growth Medium containing 5% fetal calf serum and incubated at 37°C for 4 hours.

The conditioned media from the BHK 570 transfectants were then assayed for the ability to cause cell proliferation in BaF3 cells expressing the mouse MPL

receptor. The cells were grown in BaF3 media (RPMI 1640 media (JRH Biosciences) supplemented with 10% fetal calf serum, 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, 57  $\mu$ M  $\beta$ -Mercaptoethanol, .05 mg/ml penicillin, .05 mg/ml streptomycin, .01 mg/ml neomycin and 4% V/V conditioned medium from cultures of WEHI-3 cells (mouse interleukin-3, culture supplement, Collaborative Biomedical Products)). Prior to assay, BaF3 cells were diluted and resuspended in IL-3-free BaF3 medium to 10,000 cells/100 $\mu$ l. 100  $\mu$ l of conditioned medium from pZGmpl-124 transfected BHK 570 cells was added, and the cultures were incubated at 37°C. Cells were then visually examined for cell elongation after 30 minutes and after 24 hours. A negative control consisting of BaF3 medium without IL-3 and a positive control of conditioned medium from BHK 570 cells transfected with the mouse TPO DNA were also assayed. Results showed no cell elongation of BaF3 cells in the negative control, some cell elongation in the positive control and significant cell elongation in the pZGmpl-124 transfected cells.

#### Example XV. Receptor Affinity Precipitation

150-mm tissue culture plates containing cells producing TPO or normal BHK cells were labeled for 18 hours with 10 ml of Dulbecco's MEM without methionine containing 2mM L-glutamine, antibiotics and 200  $\mu$ Ci of  $^{35}$ S-Express (Amersham, Arlington Heights, IL).

After the overnight incubation the spent media were collected and concentrated 15 times using a Centriprep-10<sup>TM</sup> concentrator (Amicon, Inc.). The resulting 0.7 ml of concentrated supernatant was mixed with 40  $\mu$ l of poly-histidine tailed soluble MPL receptor which had been linked to CNBr-Sepharose 4B (Pharmacia) as directed by the supplier. The mixture was incubated for two hours on ice, while shaking.

The cells were washed once with PBS, then lysed with 1 ml of RIP A buffer (10 mM Tris, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 0.15 M NaCl). The lysate was centrifuged to remove insoluble material, and 40  $\mu$ l of MPL-Sepharose was added as above.

The MPL-Sepharose was then pelleted by low speed centrifugation, and the spent media and cell lysate supernatants were removed. The pellet was washed four times with PBS containing 0.5 M NaCl. After the final wash, the PBS was removed, and 40  $\mu$ l of 2X sample buffer (10% glycerol, 4% SDS, 50 mM Tris, pH 7.0, 1 mM EDTA, 0.05% bromophenol blue) containing 4% beta-mercaptoethanol was added.

The samples were boiled for five minutes, and 18  $\mu$ l of each was loaded onto a 10-20% gradient mini-gel (Integrated Separation Systems), then electrophoresed at 100V for approximately two hours. The gel was fixed for thirty minutes (in 40% methanol, 16% glacial acetic acid in distilled water), then soaked in Amplify™ (Amersham) for twenty minutes. After drying, the gel was exposed to film overnight. A -70 kD band was highly visible in the lane corresponding to spent media from cells transfected with TPO cDNA. This band was not present in spent media from BHK cells or in cell lysates from either cell line.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: ZymoGenetics, Inc.  
1201 Eastlake Avenue East  
Seattle  
WA  
US  
98102

APPLICANT: University of Washington  
Seattle  
WA  
US  
98195

(ii) TITLE OF INVENTION: HEMATOPOIETIC PROTEIN AND MATERIALS AND  
METHODS FOR MAKING IT

(iii) NUMBER OF SEQUENCES: 29

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: ZymoGenetics, Inc.  
(B) STREET: 1201 Eastlake Avenue East  
(C) CITY: Seattle  
(D) STATE: WA  
(E) COUNTRY: USA  
(F) ZIP: 98102

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Parker, Gary E
- (B) REGISTRATION NUMBER: 31-648
- (C) REFERENCE/DOCKET NUMBER: 93-12PC

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 206-442-6600 ext 6673
- (B) TELEFAX: 206-442-6678

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1486 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: 1081

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 105..1241

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCGTGCCG	GTCTGAGGC	CCTTCTCCAC	CCGGACAGAG	TCCTTGCCCC	ACCTCTCTCC	60
CACCCGACTC TGCCGAAAGA AGCACAGAAG CTCAAGCCGC CTCC ATG GCC CCA GGA						116
Met Ala Pro Gly						
1						
AAG ATT CAG GGG AGA GGC CCC ATA CAG GGA GCC ACT TCA GTT AGA CAC	164					
Lys Ile Gln Gly Arg Gly Pro Ile Gln Gly Ala Thr Ser Val Arg His						
5 10 15 20						
CTG GCC AGA ATG GAG CTG ACT GAT TTG CTC CTG GCG GCC ATG CTT CTT						212
Leu Ala Arg Met Glu Leu Thr Asp Leu Leu Leu Ala Ala Met Leu Leu						
25 30 35						



GCA GTG GCA AGA CTA ACT CTG TCC AGC CCC GTA GCT CCT GCC TGT GAC	260
Ala Val Ala Arg Leu Thr Leu Ser Ser Pro Val Ala Pro Ala Cys Asp	
40 45 50	
CCC AGA CTC CTA AAT AAA CTG CTG CGT GAC TCC CAC CTC CTT CAC AGC	308
Pro Arg Leu Leu Asn Lys Leu Leu Arg Asp Ser His Leu Leu His Ser	
55 60 65	
CGA CTG AGT CAG TGT CCC GAC GTC GAC CCT TTG TCT ATC CCT GTT CTG	356
Arg Leu Ser Gln Cys Pro Asp Val Asp Pro Leu Ser Ile Pro Val Leu	
70 75 80	
CTG CCT GCT GTG GAC TTT AGC CTG GGA GAA TGG AAA ACC CAG ACG GAA	404
Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Thr Glu	
85 90 95 100	
CAG AGC AAG GCA CAG GAC ATT CTA GGG GCA GTG TCC CTT CTA CTG GAG	452
Gln Ser Lys Ala Gln Asp Ile Leu Gly Ala Val Ser Leu Leu Leu Glu	
105 110 115	
GGA GTG ATG GCA GCA CGA GGA CAG TTG GAA CCC TCC TGC CTC TCA TCC	500
Gly Val Met Ala Ala Arg Gly Gln Leu Glu Pro Ser Cys Leu Ser Ser	
120 125 130	
CTC CTG GGA CAG CTT TCT GGG CAG GTT CGC CTC CTC TTG GGG GCC CTG	548
Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu	
135 140 145	
CAG GGC CTC CTA GGA ACC CAG CTT CCT CTA CAG GGC AGG ACC ACA GCT	596
Gln Gly Leu Leu Gly Thr Gln Leu Pro Leu Gln Gly Arg Thr Thr Ala	
150 155 160	
CAC AAG GAC CCC AAT GCC CTC TTC TTG AGC TTG CAA CAA CTG CTT CGG	644
His Lys Asp Pro Asn Ala Leu Phe Leu Ser Leu Gln Gln Leu Leu Arg	
165 170 175 180	
GGA AAG GTG CGC TTC CTG CTT CTG GTA GAA GGT CCC ACC CTC TGT GTC	692
Gly Lys Val Arg Phe Leu Leu Leu Val Glu Gly Pro Thr Leu Cys Val	
185 190 195	
AGA CGG ACC CTG CCA ACC ACA GCT GTC CCA AGC AGT ACT TCT CAA CTC	740
Arg Arg Thr Leu Pro Thr Thr Ala Val Pro Ser Ser Thr Ser Gln Leu	
200 205 210	

CTC ACA CTA AAC AAG TTC CCA AAC AGG ACT TCT GGA TTG TTG GAG ACG Leu Thr Leu Asn Lys Phe Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr 215 220 225	788
AAC TTC AGT GTC ACA GCC AGA ACT GCT GGC CCT GGA CTT CTG AGC AGG Asn Phe Ser Val Thr Ala Arg Thr Ala Gly Pro Gly Leu Leu Ser Arg 230 235 240	836
CTT CAG GGA TTC AGA GTC AAG ATT ACT CCT GGT CAG CTA AAT CAA ACC Leu Gln Gly Phe Arg Val Lys Ile Thr Pro Gly Gln Leu Asn Gln Thr 245 250 255 260	884
TCC AGG TCC CCA GTC CAA ATC TCT GGA TAC CTG AAC AGG ACA CAC GGA Ser Arg Ser Pro Val Gln Ile Ser Gly Tyr Leu Asn Arg Thr His Gly 265 270 275	932
CCT GTG AAT GGA ACT CAT GGG CTC TTT GCT GGA ACC TCA CTT CAG ACC Pro Val Asn Gly Thr His Gly Leu Phe Ala Gly Thr Ser Leu Gln Thr 280 285 290	980
CTG GAA GCC TCA GAC ATC TCG CCC GGA GCT TTC AAC AAA GGC TCC CTG Leu Glu Ala Ser Asp Ile Ser Pro Gly Ala Phe Asn Lys Gly Ser Leu 295 300 305	1028
GCA TTC AAC CTC CAG GGT GGA CTT CCT CCT TCT CCA AGC CTT GCT CCT Ala Phe Asn Leu Gln Gly Gly Leu Pro Pro Ser Pro Ser Leu Ala Pro 310 315 320	1076
GAT GGA CAC ACA CCC TTC CCT CCT TCA CCT GCC TTG CCC ACC ACC CAT Asp Gly His Thr Pro Phe Pro Pro Ser Pro Ala Leu Pro Thr Thr His 325 330 335 340	1124
GGA TCT CCA CCC CAG CTC CAC CCC CTG TTT CCT GAC CCT TCC ACC ACC Gly Ser Pro Pro Gln Leu His Pro Leu Phe Pro Asp Pro Ser Thr Thr 345 350 355	1172
ATG CCT AAC TCT ACC GCC CCT CAT CCA GTC ACA ATG TAC CCT CAT CCC Met Pro Asn Ser Thr Ala Pro His Pro Val Thr Met Tyr Pro His Pro 360 365 370	1220
AGG AAT TTG TCT CAG GAA ACA TAGCGCGGGC ACTGGCCCAG TGAGCGTCTG Arg Asn Leu Ser Gln Glu Thr 375	1271

CAGCTTCTCT CGGGGACAAG CTTCCCCAGG AAGGCTGAGA GGCAGCTGCA TCTGCTCCAG 1331  
 ATGTTCTGCT TTCACCTAAA AGGCCCTGGG GAAGGGATAC ACAGCACTGG AGATTGTAAA 1391  
 ATTTTAGGAG CTATTTTTTT TTAACCTATC AGCAATATTC ATCAGAGCAG CTAGCGATCT 1451  
 TTGGTCTATT TTCGGTATAA ATTTGAAAAT CACTA 1486

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 379 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Gly Lys Ile Gln Gly Arg Gly Pro Ile Gln Gly Ala Thr  
 1 5 10 15  
 Ser Val Arg His Leu Ala Arg Met Glu Leu Thr Asp Leu Leu Leu Ala  
 20 25 30  
 Ala Met Leu Leu Ala Val Ala Arg Leu Thr Leu Ser Ser Pro Val Ala  
 35 40 45  
 Pro Ala Cys Asp Pro Arg Leu Leu Asn Lys Leu Leu Arg Asp Ser His  
 50 55 60  
 Leu Leu His Ser Arg Leu Ser Gln Cys Pro Asp Val Asp Pro Leu Ser  
 65 70 75 80  
 Ile Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys  
 85 90 95  
 Thr Gln Thr Glu Gln Ser Lys Ala Gln Asp Ile Leu Gly Ala Val Ser  
 100 105 110  
 Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Glu Pro Ser  
 115 120 125

Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu  
 130 135 140

Leu Gly Ala Leu Gln Gly Leu Leu Gly Thr Gln Leu Pro Leu Gln Gly  
 145 150 155 160

Arg Thr Thr Ala His Lys Asp Pro Asn Ala Leu Phe Leu Ser Leu Gln  
 165 170 175

Gln Leu Leu Arg Gly Lys Val Arg Phe Leu Leu Leu Val Glu Gly Pro  
 180 185 190

Thr Leu Cys Val Arg Arg Thr Leu Pro Thr Thr Ala Val Pro Ser Ser  
 195 200 205

Thr Ser Gln Leu Leu Thr Leu Asn Lys Phe Pro Asn Arg Thr Ser Gly  
 210 215 220

Leu Leu Glu Thr Asn Phe Ser Val Thr Ala Arg Thr Ala Gly Pro Gly  
 225 230 235 240

Leu Leu Ser Arg Leu Gln Gly Phe Arg Val Lys Ile Thr Pro Gly Gln  
 245 250 255

Leu Asn Gln Thr Ser Arg Ser Pro Val Gln Ile Ser Gly Tyr Leu Asn  
 260 265 270

Arg Thr His Gly Pro Val Asn Gly Thr His Gly Leu Phe Ala Gly Thr  
 275 280 285

Ser Leu Gln Thr Leu Glu Ala Ser Asp Ile Ser Pro Gly Ala Phe Asn  
 290 295 300

Lys Gly Ser Leu Ala Phe Asn Leu Gln Gly Gly Leu Pro Pro Ser Pro  
 305 310 315 320

Ser Leu Ala Pro Asp Gly His Thr Pro Phe Pro Pro Ser Pro Ala Leu  
 325 330 335

Pro Thr Thr His Gly Ser Pro Pro Gln Leu His Pro Leu Phe Pro Asp  
 340 345 350

Pro Ser Thr Thr Met Pro Asn Ser Thr Ala Pro His Pro Val Thr Met  
 355 360 365

98

Tyr Pro His Pro Arg Asn Leu Ser Gln Glu Thr  
370 375

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC5499

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGAGCCACTT TCTGCACTCC TCGAGTTTTT TTTTTTTTTT TT

42

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC5746

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGAGAGAGA GAGAATTCAT GCCCTCCTGG GCCCTCTTCA TGGTC

45

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 52 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC5762

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGAGAGAGAG AGAGCTCGAG TCAAGGCTGC TGCCAATAGC TTAGTGGTAG GT

52

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC5742

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACCCTGGAG CTGCGCCCGC GATCTCGCTA

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6091

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCACAGAA TTCACTACTC GAGGCGGCCG CTTTTTTTTT TTTTTTTTTT

49

(2) INFORMATION FOR SEQ ID NO:8:

100

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC6603

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGGAATTG CAGAAGCCAT GCCCTCTTG GCCCTCTCA TGGTC

45

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val	Arg	Thr	Ser	Pro	Ala	Gly	Glu
1				5			

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC6704

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGAGGAAT TCACCATGGA TGTCTTCTTG CTGGCCTTGG GCACAGAG

48

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 60 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC6703

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGACTTTACC TCGAGTGCTA CTGATGCTCT TCTGCCAGCA GTCTCGGAGC CCGTGGACAC 60

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 42 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC6707

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCGCCAT GGGACTCGAG CATCACCATC ACCATCACTG AG 42

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 42 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear



102

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6706

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCCTCAGT GATGGTGATG GTGATGCTCG AGTCCCATGG CG

42

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 47 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6172

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCGGTGCTC AGCATTCACT ACTCGAGGGT TTTTTTTTTT TTTTTT

47

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6936

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTGGCGGC CGCGTCGACT CGTGGATG

28

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 28 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
 (B) CLONE: ZC6937

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTCATCCA CGAGTCGACG CGGCCGCC

28

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 633 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala  
 1 5 10 15

Leu Pro Asn Gln Ala Gln Val Thr Ser Gln Asp Val Phe Leu Leu Ala  
 20 25 30

Leu Gly Thr Glu Pro Leu Asn Cys Phe Ser Gln Thr Phe Glu Asp Leu  
 35 40 45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln  
 50 55 60

Leu Leu Tyr Ala Tyr Arg Gly Glu Lys Pro Arg Ala Cys Pro Leu Tyr  
 65 70 75 80

104

Ser Gln Ser Val Pro Thr Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro  
 85 90 95  
 Ala Gln Asp Glu Val Arg Leu Phe Phe Pro Leu His Leu Trp Val Lys  
 100 105 110  
 Asn Val Ser Leu Asn Gln Thr Leu Ile Gln Arg Val Leu Phe Val Asp  
 115 120 125  
 Ser Val Gly Leu Pro Ala Pro Pro Arg Val Ile Lys Ala Arg Gly Gly  
 130 135 140  
 Ser Gln Pro Gly Glu Leu Gln Ile His Trp Glu Ala Pro Ala Pro Glu  
 145 150 155 160  
 Ile Ser Asp Phe Leu Arg His Glu Leu Arg Tyr Gly Pro Thr Asp Ser  
 165 170 175  
 Ser Asn Ala Thr Ala Pro Ser Val Ile Gln Leu Leu Ser Thr Glu Thr  
 180 185 190  
 Cys Cys Pro Thr Leu Trp Met Pro Asn Pro Val Pro Val Leu Asp Gln  
 195 200 205  
 Pro Pro Cys Val His Pro Thr Ala Ser Gln Pro His Gly Pro Val Arg  
 210 215 220  
 Thr Ser Pro Ala Gly Glu Ala Pro Phe Leu Thr Val Lys Gly Gly Ser  
 225 230 235 240  
 Cys Leu Val Ser Gly Leu Gln Ala Gly Lys Ser Tyr Trp Leu Gln Leu  
 245 250 255  
 Arg Ser Gln Pro Asp Gly Val Ser Leu Arg Gly Ser Trp Gly Pro Trp  
 260 265 270  
 Ser Phe Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Thr Ile Gly  
 275 280 285  
 Leu Gln Cys Phe Thr Leu Asp Leu Lys Met Val Thr Cys Gln Trp Gln  
 290 295 300  
 Gln Gln Asp Arg Thr Ser Ser Gln Gly Phe Phe Arg His Ser Arg Thr  
 305 310 315 320

105

Arg Cys Cys Pro Thr Asp Arg Asp Pro Thr Trp Glu Lys Cys Glu Glu  
 325 330 335  
 Glu Glu Pro Arg Pro Gly Ser Gln Pro Ala Leu Val Ser Arg Cys His  
 340 345 350  
 Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val Thr  
 355 360 365  
 Thr Ala Gln Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp Ile  
 370 375 380  
 His Gln Ala Val Leu Leu Pro Thr Pro Ser Leu His Trp Arg Glu Val  
 385 390 395 400  
 Ser Ser Gly Arg Leu Glu Leu Glu Trp Gln His Gln Ser Ser Trp Ala  
 405 410 415  
 Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly Arg Glu  
 420 425 430  
 Asp Trp Lys Val Leu Glu Pro Ser Leu Gly Ala Arg Gly Gly Thr Leu  
 435 440 445  
 Glu Leu Arg Pro Arg Ala Arg Tyr Ser Leu Gln Leu Arg Ala Arg Leu  
 450 455 460  
 Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ala Trp Ser Pro Pro Ala  
 465 470 475 480  
 Arg Val Ser Thr Gly Ser Glu Thr Ala Trp Ile Thr Leu Val Thr Ala  
 485 490 495  
 Leu Leu Leu Val Leu Ser Leu Ser Ala Leu Leu Gly Leu Leu Leu Leu  
 500 505 510  
 Lys Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu Trp  
 515 520 525  
 Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg Asp  
 530 535 540  
 Thr Ala Ala Leu Ser Pro Ser Lys Ala Thr Val Thr Asp Ser Cys Glu  
 545 550 555 560



107

CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT GTC CTG CTG CCT GCT Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala 50 55 60	192
GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC CAG ATG GAG GAG ACC AAG Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys 65 70 75 80	240
GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met 85 90 95	288
GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly 100 105 110	336
CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu 115 120 125	384
CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp 130 135 140	432
CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val 145 150 155 160	480
CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTC AGG CGG GCC Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala 165 170 175	528
CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC TCT CTA GTC CTC ACA CTG Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu 180 185 190	576
AAC GAG CTC CCA AAC AGG ACT TCT GGA TTG TTG GAG ACA AAC TTC ACT Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr 195 200 205	624
GCC TCA GCC AGA ACT ACT GGC TCT GGG CTT CTG AAG TGG CAG CAG GGA Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly 210 215 220	672

TTC AGA GCC AAG ATT CCT GGT CTG CTG AAC CAA ACC TCC AGG TCC CTG Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu 225 230 235 240	720
GAC CAA ATC CCC GGA TAC CTG AAC AGG ATA CAC GAA CTC TTG AAT GGA Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly 245 250 255	768
ACT CGT GGA CTC TTT CCT GGA CCC TCA CGC AGG ACC CTA GGA GCC CCG Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro 260 265 270	816
GAC ATT TCC TCA GGA ACA TCA GAC ACA GGC TCC CTG CCA CCC AAC CTC Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu 275 280 285	864
CAG CCT GGA TAT TCT CCT TCC CCA ACC CAT CCT CCT ACT GGA CAG TAT Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr 290 295 300	912
ACG CTC TTC CCT CTT CCA CCC ACC TTG CCC ACC CCT GTG GTC CAG CTC Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu 305 310 315 320	960
CAC CCC CTG CTT CCT GAC CCT TCT GCT CCA ACG CCC ACC CCT ACC AGC His Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser 325 330 335	1008
CCT CTT CTA AAC ACA TCC TAC ACC CAC TCC CAG AAT CTG TCT CAG GAA Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu 340 345 350	1056
GGG TAA Gly	1062

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 353 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Glu Leu Thr Glu Leu Leu Val Val Met Leu Leu Leu Thr Ala  
 1 5 10 15  
 Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val  
 20 25 30  
 Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser  
 35 40 45  
 Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala  
 50 55 60  
 Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys  
 65 70 75 80  
 Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met  
 85 90 95  
 Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
 100 105 110  
 Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
 115 120 125  
 Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
 130 135 140  
 Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
 145 150 155 160  
 Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala  
 165 170 175  
 Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu  
 180 185 190  
 Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr  
 195 200 205  
 Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly  
 210 215 220



110

Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu  
 225                      230                      235                      240  
 Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly  
                     245                      250                      255  
 Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro  
                     260                      265                      270  
 Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu  
                     275                      280                      285  
 Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr  
                     290                      295                      300  
 Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu  
 305                      310                      315                      320  
 His Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser  
                     325                      330                      335  
 Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu  
                     340                      345                      350

Gly

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:
- (B) CLONE: ZC7422

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGAAGCTGGG TACCAAGGAG GCT

111

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 23 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC7423

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCCTCCTTG GTACCCAGCT TCC

23

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 20 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC7424

## (x1) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTAGACACCT GGCCAGAATG

20

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 24 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC7421

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGATGTCGGC AGTGTCTGAG AACC

24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC7454

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCGGAATTCT TAGACACCTG GCCAGAATG

29

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC7453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGGAATTCT GATGTCGGCA GTGTCTGAGA ACC

33

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid

113

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: ZC7318

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TACCGAATTC TAGACACAGA GGGTGGGACC TTC

33

- (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: ZC7319

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ACACTGAATT CTTCTCCACC CGGACAGAGT

30

- (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 4823 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(632..644, 876..1003, 1290..1376, 3309..3476, 3713..4375)

114

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTTCTTGCT TTCTTTCTT CTTCTTTCT TTCTTTTTT TTTTGAGAC GGAGTTTCAC	60
TCTTATTGCC CAGGCTGGAG TGCAATGGT CGATCTCGGC TCACCACAAC CTCCGCCTCC	120
CAGGTACAAG CGATTCTCCT GTCTCAGCCT CCCAAGTAGC TTGGATTACA GGCATGAACC	180
ACCACACCCT GCTAGTTTTT TTGTATTTG TAGAGCCGGG GTTTCACCAT GTTAGTGAGG	240
CTGGTGGCGA ACTCCTGACC TCAGGTGATC CACCCGCCTT GGACTCCCAA AGTGCTGGGA	300
TTACAGGCAT GAGCCACTGC ACCCGGCACA CCATATGCTT TCATCACAAG AAAATGTGAG	360
AGAATTCAAG GCTTTGGCAG TTCCAGGCTG GTCAGCATCT CAAGCCCTCC CCAGCATCTG	420
TTCACCCTGC CAGGCAGTCT CTTCTAGAA ACTTGGTAA ATGTTCACTC TTCTTGCTAC	480
TTTCAGGATA GATTCTTCAC CTTGGTCCG CCTTTGCCCC ACCCTACTCT GCCCAGAAGT	540
GCAAGAGCCT AAGCCGCTC CATGGCCCCA GGAAGGATTC AGGGGAGAGG CCCCAAACAG	600
GGAGCCACGC CAGCCAGACA CCCCAGCCAG A ATG GAG CTG ACT G GTGAGAACAC	654
Met Glu Leu Thr	
1	
ACCTGAGGGG CTAGGGCCAT ATGGAACAT GACAGAAGGG GAGAGAGAAA GGAGACACGC	714
TGCAGGGGGC AGGAAGCTGG GGAACCCAT TCTCCAAAA ATAAGGGGTC TGAGGGGTGG	774
ATTCCTGGG TTTCAGGTCT GGGTCCTGAA TGGGAATTCC TGGGAATACCA GCTGACAATG	834
ATTCCTCCT CATCTTCAA C CTCACCTCT CCTCATCTAA G AA TTG CTC CTC	886
Glu Leu Leu Leu	
5	
GTG GTC ATG CTT CTC CTA ACT GCA AGG CTA ACG CTG TCC AGC CCG GCT	934
Val Val Met Leu Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro Ala	
10 15 20	
CCT CCT GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC	982
Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser	
25 30 35 40	

115

CAT GTC CTT CAC AGC AGA CTG GTGAGAACTC CCAACATTAT CCCCTTTATC His Val Leu His Ser Arg Leu 45	1033
CGCGTAACTG GTAAGACACC CATACTCCCA GGAAGACACC ATCACTTCCT CTAACCTCCT	1093
GACCCAATGA CTATTCTTCC CATATTGTCC CCACCTACTG ATCACACTCT CTGACAAGGA	1153
TTATTCTTCA CAATACAGCC CGCATTTAAA AGCTCTCGTC TAGAGATAGT ACTCATGGAG	1213
GACTAGCCTG CTTATTAGGC TACCATAGCT CTCTCTATTT CAGCTCCCTT CTCCCCCAC	1273
CAATCTTTTT CAACAG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr 50 55	1322
CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr 60 65 70	1370
CAG ATG GTAAGAAAGC CATCCCTAAC CTGGGCTTCC CTAAGTCCTG TCTTCAGTT Gln Met 75	1426
CCCACTGCTT CCCATGGATT CTCCAACATT CTTGAGCTTT TTAAAAATAT CTCACCTTCA	1486
GCTTGGCCAC CCTAACCCAA TCTACATTCA CCTATGATGA TAGCCTGTGG ATAAGATGAT	1546
GGCTTGCAGG TCCAATATGT GAATAGATTT GAAGCTGAAC ACCATGAAAA GCTGGAGAGA	1606
AATCGCTCAT GGCCATGCCT TTGACCTATT CCCGTTCACT CTTCTTAAAT TGGCATGAAG	1666
AAGCAAGACT CATATGTCAT CCACAGATGA CACAAAGCTG GGAAGTACCA CTAAAATAAC	1726
AAAAGACTGA ATCAAGATTC AAATCACTGA AAGACTAGGT CAAAACAAG GTGAAACAAC	1786
AGAGATATAA ACTTCTACAT GTGGGCCGGG GGCTCACGCC TGTAAATCCA GCACTTTGGG	1846
AGGCCGAGGC AGGCAGATCA CCTGAGGGCA GGAGTTTGAG AGCAGCCTGG CCAACATGGC	1906
GAAACCCCGT CTCTACTAAG AATACAGAAT TAGCCGGGCA TGGTAGTGCA TGCCTGTAAT	1966
CCCAGCTACT TGAAGGCTG AAGCAGGAGA ATCCCTTGAA CCCAGGAGGT GGAGGTGTA	2026
GTGAGCTGAG ATCATGCCAA TGCACTCCAG CCTGGGTGAC AAGAGCAAAA CTCCGTCTCA	2086

AAAAGAAAA AAAATTCTAC ATGTGTAAAT TAATGAGTAA AGTCCTATTC CAGCTTTCAG	2146
GCCACAATGC CCTGCTTCCA TCATTTAAGC CTCTGGCCCT AGCACTTCCT ACGAAAAGGA	2206
TCTGAGAGAA TTAAATTGCC CCCAACTTA CCATGTAACA TTAAGAAGC TGCTATTCTT	2266
AAAGCTAGTA ATTCTTGCTT GTTTGATGTT TAGCATCCCC ATTGTGGAAA TGCTCGTACA	2326
GAACTCTATT CCGAGTGGAC TACACTTAAA TATACTGGCC TGAACACCGG ACATCCCCCT	2386
GAAGACATAT GCTAATTTAT TAAGAGGGAC CATATTAAAC TAACATGTGT CTAGAAAGCA	2446
GCAGCCTGAA CAGAAAGAGA CTAGAAGCAT GTTTTATGGG CAATAGTTTA AAAAATAAAA	2506
ATCTATCCTC AAGAACCCTA GCGTCCCTTC TTCCTTCAGG ACTGAGTCAG GGAAGAAGGG	2566
CAGTTCCTAT GGGTCCCTTC TAGTCCTTTC TTTTCATCCT TATGATCATT ATGGTAGAGT	2626
CTCATACCTA CATTTAGTTT ATTTATTATT ATTATTTGAG ACGGAGTCTC ACTCTATCCC	2686
CCAGGCTGGA GTGCAGTGGC ATGATCTCAA CTCAGTCAA CCTCAGCCTC CCGGATTCAA	2746
GCATTCTCC TGTCTCAGTC TCCCAAGTAG CTGGGATTAC AGGTGCCAC CACCATGCCC	2806
AGCTAATTTG TGTATTTGTG GTAGAGATGG GGTTCACCA TGTTGGGCAG GCTGATCTTG	2866
AACTCCTGAC CTCAGGTGAT CCACCTGCCT CAGCCTCCA AAGTGCTGGG ATTACAGGCG	2926
TGAGCCACTG CACCCAGCCT TCATTCAGTT TAAAAATCAA ATGATCCTAA GGTTTTGCAG	2986
CAGAAAGAGT AAATTTGCAG CACTAGAACC AAGAGGTAAA AGCTGTAACA GGGCAGATTT	3046
CAGCAACGTA AGAAAAAGG AGCTCTTCTC ACTGAAACCA AGTGTAAGAC CAGGCTGGAC	3106
TAGAGGACAC GGGAGTTTTT GAAGCAGAGG CTGATGACCA GCTGTCGGGA GACTGTGAAG	3166
GAATTCCTGC CCTGGGTGGG ACCTTGGTCC TGTCCAGTTC TCAGCCTGTA TGATTCACCTC	3226
TGCTGGCTAC TCCTAAGGCT CCCACCCGC TTTTAGTGTG CCCTTTGAGG CAGTGCGCTT	3286
CTCTCTTCCA TCTCTTCTC AG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA	3338
Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly	
80 85	

117

GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu 90 95 100	3386
GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val 105 110 115	3434
CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln 120 125 130	3476
GTAAGTCCCC AGTCAAGGGA TCTGTAGAAA CTGTTCTTTT CTGACTCAGT CCCCCTAGAA	3536
GACCTGAGGG AAGAAGGGCT CTTCCAGGGA GCTCAAGGGC AGAAGAGCTG ATCTACTAAG	3596
AGTGCTCCCT GCCAGCCACA ATGCCTGGGT ACTGGCATCC TGTCTTTCCT ACTTAGACAA	3656
GGGAGGCCTG AGATCTGGCC CTGGTGTGTTG GCCTCAGGAC CATCCTCTGC CCTCAG	3712
CTT CCT CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT CCC AAT GCC ATC Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile 135 140 145	3760
TTC CTG AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG CGT TTC CTG ATG Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met 150 155 160	3808
CTT GTA GGA GGG TCC ACC CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr 165 170 175 180	3856
GCT GTC CCC AGC AGA ACC TCT CTA GTC CTC ACA CTG AAC GAG CTC CCA Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu Pro 185 190 195	3904
AAC AGG ACT TCT GGA TTG TTG GAG ACA AAC TTC ACT GCC TCA GCC AGA Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg 200 205 210	3952
ACT ACT GGC TCT GGG CTT CTG AAG TGG CAG CAG GGA TTC AGA GCC AAG Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys 215 220 225	4000



ATT CCT GGT CTG CTG AAC CAA ACC TCC AGG TCC CTG GAC CAA ATC CCC Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro 230 235 240	4048
GGA TAC CTG AAC AGG ATA CAC GAA CTC TTG AAT GGA ACT CGT GGA CTC Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu 245 250 255 260	4096
TTT CCT GGA CCC TCA CGC AGG ACC CTA GGA GCC CCG GAC ATT TCC TCA Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser 265 270 275	4144
GGA ACA TCA GAC ACA GGC TCC CTG CCA CCC AAC CTC CAG CCT GGA TAT Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr 280 285 290	4192
TCT CCT TCC CCA ACC CAT CCT CCT ACT GGA CAG TAT ACG CTC TTC CCT Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro 295 300 305	4240
CTT CCA CCC ACC TTG CCC ACC CCT GTG GTC CAG CTC CAC CCC CTG CTT Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu His Pro Leu Leu 310 315 320	4288
CCT GAC CCT TCT GCT CCA ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn 325 330 335 340	4336
ACA TCC TAC ACC CAC TCC CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly 345 350	4385
AGACACTGCC GACATCAGCA TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGC GCCCTG	4445
GGAGACAACT GGACAAGATT TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA	4505
TACACAGGAC TGAAAAGGGA ATCATTTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT	4565
TTTTTAAGCT ATCAGCAATA CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTTCTGCAGA	4625
AATTTGCAAC TCACTGATTC TCAACATGCT CTTTTTCTGT GATAACTCTG CAAAGACCTG	4685
GGCTGGCCTG GCAGTTGAAC AGAGGGAGAG ACTAACCTTG AGTCAGAAAA CAGAGGAAGG	4745
GTAATTTCTT TTGCTTCAAA TTCAAGGCCT TCCAACGCCC CCATCCCCTT TACTATCATT	4805

CTCAGTGGGA CTCTGATC

4823

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 353 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Met	Glu	Leu	Thr	Glu	Leu	Leu	Leu	Val	Val	Met	Leu	Leu	Leu	Thr	Ala	1	5	10	15
Arg	Leu	Thr	Leu	Ser	Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	20	25	30	
Leu	Ser	Lys	Leu	Leu	Arg	Asp	Ser	His	Val	Leu	His	Ser	Arg	Leu	Ser	35	40	45	
Gln	Cys	Pro	Glu	Val	His	Pro	Leu	Pro	Thr	Pro	Val	Leu	Leu	Pro	Ala	50	55	60	
Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	Met	Glu	Glu	Thr	Lys	65	70	75	80
Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu	Leu	Leu	Glu	Gly	Val	Met	85	90	95	
Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr	Cys	Leu	Ser	Ser	Leu	Leu	Gly	100	105	110	
Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln	Ser	Leu	115	120	125	
Leu	Gly	Thr	Gln	Leu	Pro	Pro	Gln	Gly	Arg	Thr	Thr	Ala	His	Lys	Asp	130	135	140	

120

Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
 145                      150                      155                      160

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala  
                     165                      170                      175

Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu  
                     180                      185                      190

Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr  
                     195                      200                      205

Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly  
                     210                      215                      220

Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu  
 225                      230                      235                      240

Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly  
                     245                      250                      255

Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro  
                     260                      265                      270

Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu  
                     275                      280                      285

Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr  
                     290                      295                      300

Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu  
 305                      310                      315                      320

His Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser  
                     325                      330                      335

Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu  
                     340                      345                      350

Gly

Claims

We claim:

1. An isolated protein selected from the group consisting of:

a) proteins comprising the sequence of amino acids of SEQ ID NO: 2 from amino acid residue 45 to amino acid residue 196;

b) proteins comprising the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 206;

c) proteins comprising the sequence of amino acids of SEQ ID NO: 19 from amino acid residue 22 to amino acid residue 173;

d) proteins comprising the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 175;

e) allelic variants of (a), (b), (c) or (d); and

f) species homologs of (a), (b), (c), (d) or (e) wherein said protein stimulates proliferation or differentiation of myeloid or lymphoid precursors.

2. An isolated protein according to claim 1, wherein said protein comprises the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 379.

3. An isolated protein according to claim 1, wherein said protein comprises the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 353.

4. An isolated protein according to claim 1 wherein said protein is a mouse protein.

5. An isolated protein according to claim 1 wherein said protein is a human protein.

6. An isolated protein according to claim 1, wherein said protein comprises:

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 45 to residue 379;

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 24 to residue 196;

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 24 to residue 206;

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 24 to residue 379;

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 1 to residue 196;

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 1 to residue 206; or

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 1 to residue 379.

7. An isolated protein according to claim 1, wherein said protein comprises:

the sequence of amino acids shown in SEQ ID NO: 19 from amino acid residue 1 to residue 173;

the sequence of amino acids shown in SEQ ID NO: 19 from amino acid residue 1 to residue 175;

the sequence of amino acids shown in SEQ ID NO: 19 from amino acid residue 1 to residue 353; or

the sequence of amino acids shown in SEQ ID NO: 19 from amino acid residue 22 to residue 353.

8. An isolated protein consisting essentially of a sequence of amino acids selected from the group consisting of:

the sequence of amino acids shown in SEQ ID NO: 2 from amino acid residue 45 to amino acid residue 196;

the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 206;

the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 379;

the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 175; and

the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 353.

9. An isolated protein that stimulates the proliferation or differentiation of myeloid or lymphoid precursors, wherein said protein comprises a segment that is at least 80% identical at the amino acid level to the sequence of amino acids of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 196 or the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 173.

10. An isolated polynucleotide molecule encoding a protein according to claim 1.

11. An isolated polynucleotide molecule according to claim 10 wherein said molecule is a DNA molecule comprising a coding strand comprising the sequence of nucleotides of SEQ ID NO:1 from nucleotide 237 to nucleotide 692.

12. An isolated polynucleotide molecule according to claim 10 wherein said molecule is a DNA molecule comprising a coding strand comprising the sequence of nucleotides of SEQ ID NO:18 from nucleotide 64 to nucleotide 519.

13. An isolated polynucleotide molecule according to claim 10 wherein said molecule encodes the amino acid sequence of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 196.

14. An isolated polynucleotide molecule according to claim 10 wherein said molecule encodes the amino acid sequence of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 173.

15. An isolated polynucleotide molecule selected from the group consisting of:

(a) DNA molecules encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 237 to nucleotide 692;

(b) DNA molecules encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:18 from nucleotide 64 to nucleotide 519;

(c) allelic variants of (a) or (b);

(d) DNA molecules encoding a hematopoietic protein that is at least 80% identical in amino acid sequence to a protein encoded by (a), (b) or (c); and

(e) molecules complementary to (a), (b), (c) or (d).

16. An isolated polynucleotide molecule according to claim 15 wherein said molecule encodes a hematopoietic protein that is at least 90% identical in amino acid sequence to a protein encoded by (a), (b) or (c).

17. An isolated polynucleotide molecule according to claim 15 wherein said molecule comprises nucleotide 237 to nucleotide 722 of SEQ ID NO: 1 or nucleotide 64 to nucleotide 525 of SEQ ID NO: 18.

18. An isolated DNA molecule selected from the group consisting of:

(a) the *Eco* RI-*Xho* I insert of plasmid pZGmpl-1081 (ATCC 69566);

(b) allelic variants of (a); and

(c) DNA molecules encoding a protein that is at least 80% identical in amino acid sequence to a protein

encoded by (a) or (b), wherein said isolated DNA molecule encodes a protein having hematopoietic activity.

19. An isolated DNA molecule according to claim 18 wherein said molecule encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 from amino acid residue 45 to amino acid residue 196.

20. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment selected from the group consisting of:

(a) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 237 to nucleotide 692;

(b) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:18 from nucleotide 64 to nucleotide 519;

(c) allelic variants of (a) or (b); and

(d) DNA segments encoding a hematopoietic protein that is at least 80% identical in amino acid sequence to a protein encoded by (a), (b) or (c); and

a transcription terminator.

21. An expression vector according to claim 20 wherein said DNA segment encodes a hematopoietic protein that is at least 90% identical in amino acid sequence to a protein encoded by (a), (b) or (c).

22. An expression vector according to claim 20 wherein said DNA segment comprises nucleotide 237 to nucleotide 722 of SEQ ID NO: 1 or nucleotide 64 to nucleotide 525 of SEQ ID NO: 18.



23. An expression vector according to claim 20 further comprising a secretory signal sequence operably linked to the DNA segment.

24. A cultured cell into which has been introduced an expression vector according to claim 20, wherein said cell expresses a hematopoietic protein encoded by the DNA segment.

25. A cultured cell according to claim 24 wherein said cell is a fungal cell.

26. A cultured cell according to claim 25 wherein said cell is a yeast cell.

27. A cultured cell according to claim 24 wherein said cell is a mammalian cell.

28. A cultured cell according to claim 24 wherein said cell is a bacterial cell.

29. A non-human mammal into the germ line of which has been introduced a heterologous DNA segment selected from the group consisting of:

(a) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:1 from nucleotide 237 to nucleotide 692;

(b) DNA segments encoding a hematopoietic protein and comprising a nucleotide sequence as shown in SEQ ID NO:18 from nucleotide 64 to nucleotide 519;

(c) allelic variants of (a) or (b); and

(d) DNA segments encoding a hematopoietic protein that is at least 80% identical in amino acid sequence to a protein encoded by (a), (b) or (c);

wherein said mammal produces the hematopoietic protein encoded by said DNA segment.

30. A non-human mammal according to claim 29 selected from the group consisting of pigs, goats, sheep, cattle and mice.

31. A non-human mammal according to claim 29 wherein said DNA segment comprises nucleotide 237 to nucleotide 722 of SEQ ID NO: 1 or nucleotide 64 to nucleotide 525 of SEQ ID NO: 18.

32. A method of producing a hematopoietic protein comprising:

culturing a cell into which has been introduced an expression vector according to claim 20, whereby said cell expresses a hematopoietic protein encoded by the DNA segment; and

recovering the hematopoietic protein.

33. A method according to claim 32 wherein said hematopoietic protein is secreted by said cell and is recovered from a medium in which said cell is cultured.

34. A pharmaceutical composition comprising a protein according to claim 1 in combination with a pharmaceutically acceptable vehicle.

35. An antibody that binds to an epitope of a protein according to claim 1.

36. A method for stimulating platelet production in a mammal comprising administering to said mammal a therapeutically effective amount of a hematopoietic protein selected from the group consisting of:

a) proteins comprising the sequence of amino acids of SEQ ID NO: 2 from amino acid residue 45 to amino acid residue 196;

(b) proteins comprising the sequence of amino acids of SEQ ID NO:19 from amino acid residue 22 to amino acid residue 173;

c) allelic variants of (a) or (b); and

d) species homologs of (a), (b) or (c),

wherein said protein stimulates proliferation or differentiation of myeloid or lymphoid precursors, in combination with a pharmaceutically acceptable vehicle.

37. A probe which comprises an oligonucleotide of at least 14 nucleotides, wherein the sequence of said oligonucleotide is at least 80% identical to a same-length portion of:

(a) SEQ ID NO: 1;

(b) SEQ ID NO: 18;

(c) SEQ ID NO: 28; or

(d) sequences complementary to SEQ ID NO: 1, SEQ ID NO: 18 or SEQ ID NO: 28.

38. A method for detecting, in a mixture of DNA molecules, a DNA molecule encoding thrombopoietin comprising probing a mixture of DNA molecules with a probe which comprises an oligonucleotide of at least 14 nucleotides, wherein the sequence of said oligonucleotide is at least 80% identical to a same-length portion of:

(a) SEQ ID NO: 1;

(b) SEQ ID NO: 18;

(c) SEQ ID NO: 28; or

(d) sequences complementary to SEQ ID NO: 1, SEQ ID NO: 18 or SEQ ID NO: 28; and

detecting DNA molecules to which said probe hybridizes.

39. A method for stimulating cell proliferation comprising adding to cultured bone marrow cells an isolated

protein according to claim 1 in an amount sufficient to stimulate cell proliferation.

40. A method according to claim 39 wherein said cells are megakaryocytes or megakaryocyte precursors.

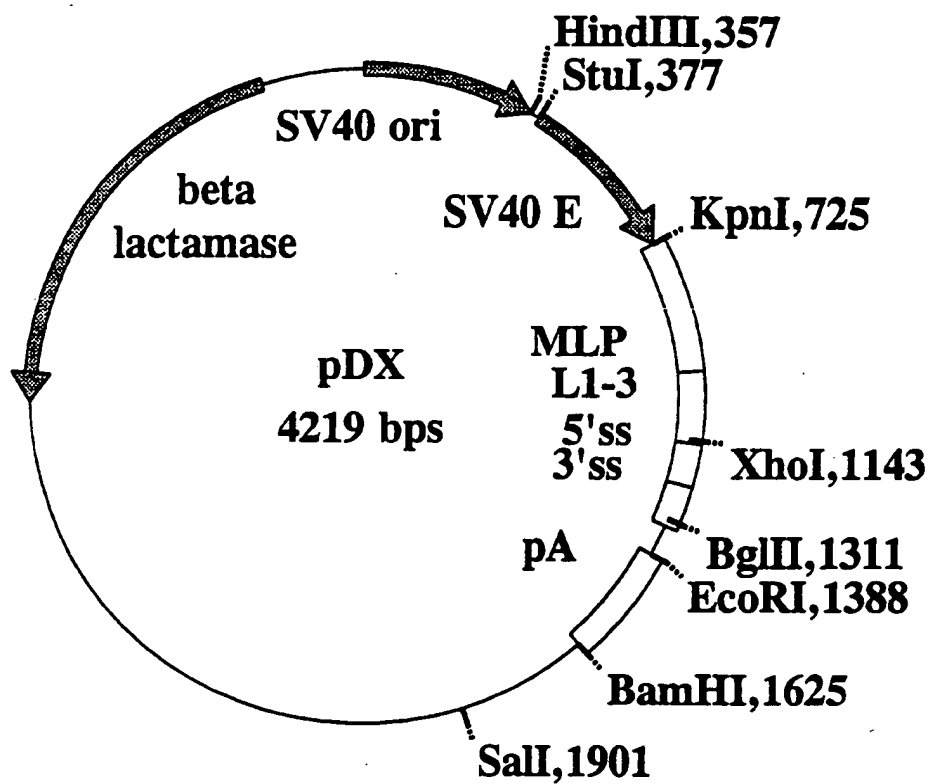
41. A method for purifying thrombopoietin comprising:

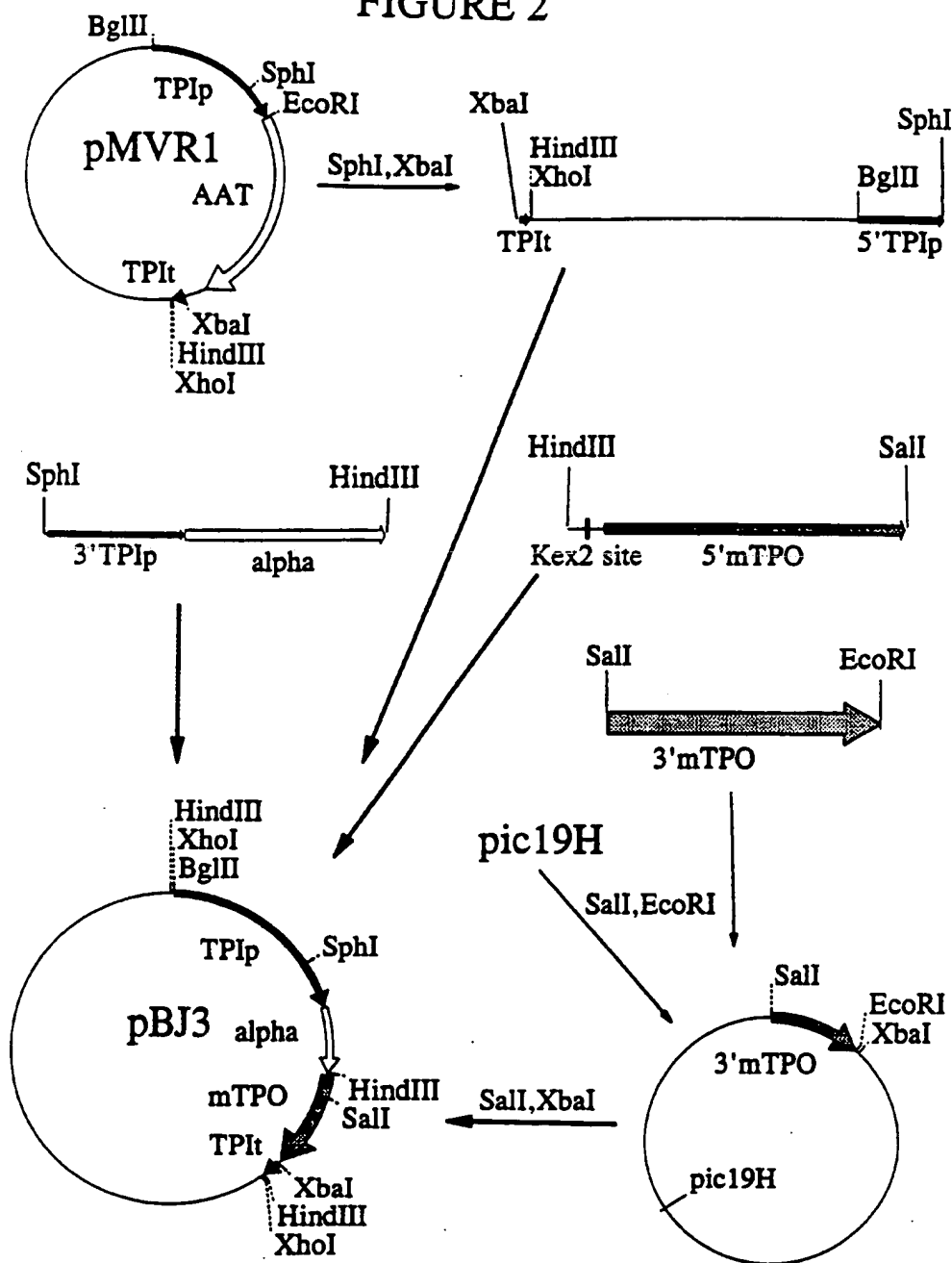
exposing a solution containing thrombopoietin to an antibody attached to a solid support, wherein said antibody binds to an epitope of a protein according to claim 1;

washing said antibody to remove unbound contaminants;

eluting bound thrombopoietin from said antibody; and  
recovering said eluted thrombopoietin.

1/2

**FIGURE 1**

2 / 2  
FIGURE 2

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 94/08806

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/19 C07K14/52 C07K16/24 C12N1/21 C12N5/10  
C12N1/19 A01K67/027 A61K38/19 C12Q1/68 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EXPERIMENTAL HEMATOLOGY, vol.17, no.8, September 1989 pages 865 - 871 T.P. MCDONALD ET AL 'A four-step procedure for the purification of thrombopoietin' see the whole document ----	1,2,5,6, 8,39,40
A	EXPERIMENTAL HEMATOLOGY, vol.16, no.3, March 1988 pages 201 - 205 T.P. MCDONALD 'Thrombopoietin : Its biology, purification and characterization' cited in the application ----- -/--	

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
 "&" document member of the same patent family

Date of the actual completion of the international search

1 December 1994

Date of mailing of the international search report

14-12-1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 631 epo nl,  
 Fax (+31-70) 340-3016

Authorized officer

Le Cornec, N

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 94/08806

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE AMERICAN JOURNAL OF PEDIATRIC HEMATOLOGY /ONCOLOGY, vol.14, no.1, 1992 pages 8 - 21 T. P. MACDONALD 'Thrombopoietin its biology, clinical aspects and possibilities' cited in the application see the whole document ---	1-9,34, 36,39,40
A	CHEMICAL ABSTRACTS, vol. 105, no. 3, 21 July 1986, Columbus, Ohio, US; abstract no. 18646m, T.P. MCDONALD ET AL 'Monoclonal antibodies to human urinary thrombopoietin' page 94 ;column L ; see abstract & PROC. SOC. EXP. BIOL. MED., vol.182, no.2, 1986 pages 151 - 158 ---	1,35,36
P,X	NATURE, vol.369, 16 June 1994, LONDON GB pages 565 - 568 S. LOK ET AL 'Cloning and expression of murine Thrombopoietin cDNA and stimulation of platelet production in vivo' see the whole document ---	1,2,4,6, 8-11,13, 15-24, 27, 32-34,36
P,X	CELL, vol.77, 1 July 1994, CAMBRIDGE, MA US pages 1117 - 1124 T.D. BARTLEY ET AL 'Identification and Cloning of a Megakaryocyte Growth and Development Factor that is a ligand for the cytokine receptor Mpl' * see the whole document especially figures 4,5 * ---	1-17, 19-24,27
A	CHEMICAL ABSTRACTS, vol. 113, no. 19, 5 November 1990, Columbus, Ohio, US; abstract no. 170137u, E.N. DESSYPRIS ET AL 'Thrombopoiesis-stimulating factor : its effects on megakaryocyte colony formation in vitro and its relation to human granulocyte-macrophage colony-stimulating factor' page 543 ;column R ; see abstract & EXPERIMENTAL HEMATOLOGY, vol.18, no.7, 1990 pages 754 - 757 --- -/--	1,39,40



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/08806

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 91, no. 21, 19 November 1979, Columbus, Ohio, US; abstract no. 186949y, T. ASHIZAWA 'Studies on thrombopoietin . II. Influence of thrombopoietin on colony forming unit megakaryocyte (CFU-M)' page 77 ;column L ; see abstract &amp; NIPPON KETSUEKI GAKKAI ZASSHI, vol.42, no.3, 1979 pages 496 - 504</p> <p>-----</p>	1,39,40

## INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 94/ 08806

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claim 36 is directed to a method of treatment of the human/animal body (rule 39-1 (iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.